SEEKING A MECHANISM OF ACTION FOR THE ANTI-HIV PROPERTIES OF THE CD8 ANTIVIRAL FACTOR, CAF

by

Varsha Shridhar

B.Tech, Anna University, 2005

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This thesis was presented

by

Varsha Shridhar

It was defended on

18th August 2011

and approved by

Velpandi Ayyavoo, PhD., Associate Professor, Infectious Diseases and Microbiology

Saleem Khan, PhD., Professor, Microbiology and Molecular Genetics

Ronald Montelaro, PhD., Professor, Microbiology and Molecular Genetics

Paul Robbins, PhD., Professor, Microbiology and Molecular Genetics

Dissertation Advisor: Phalguni Gupta, PhD.,Professor, Infectious Diseases and Microbiology

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Abstract

$CD8^+$ T cells can inhibit HIV-1 replication in infected $CD4^+$ T cells in a non-cytolytic manner by suppressing transcription from the viral promoter. The factor mediating this response, referred to as CD8 Antiviral Factor (CAF), and its mechanism of action, are unknown. The overall aim of this project was to elucidate the mechanisms by which CAF mediates its HIV-1 transcription suppressing effects. Towards this, we first studied the interaction of CAF with $CD4^+$ target cells; then, investigated the signaling molecules involved in the process of viral transcription suppression; and thirdly, identified a region on the HIV-1 Long Terminal Repeat (LTR) promoter necessary for CAF-mediated transcription suppression. We used exosomes secreted by $CD8^+$ T cells as a source of CAF to examine the interactions of CAF with its $CD4^+$ target cells. Exosomes interacted with the cells at their surface, within 10 minutes of addition. However, maximal suppression of transcription was seen in the cells only 12 to 16 hours after addition. These results suggest the involvement of an intracellular signaling cascade and, possibly, secondary gene expression. Previous reports, using HVS-transformed $CD8^+$ T cell lines, have shown the requirement for the intracellular signal transducer, STAT1, in CAF-mediated viral transcription suppression. However, we found that the requirement for STAT1 in the suppressive process was seen only when CAF from transformed $CD8^+$ T cells ($CAF_{transformed}$) was used. In contrast, CAF from primary $CD8^+$ T cells ($CAF_{primary}$), from HIV-1 infected

patients, suppressed HIV-1 transcription in a STAT1-independent manner. Our investigations on the region of the viral promoter necessary for suppression showed the importance of the minimal promoter region of HIV-1 LTR. This 90bp region, containing 3 SpI sites and TATA box, was sufficient for transcription suppression to occur. Hence, our studies implicate a multi-pronged mechanism of CAF action on the LTR minimal promoter, and indicate major differences between CAF from transformed and primary $CD8^+$ T cells.

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कर्मण्येवाधिकारस्ते मा फलेषु कदाचन।

मा कर्मफलहेतुर्भूर्मा ते सङ्गोस्त्वऽकर्मणि॥

(Bhagavad Gita, Chapter 2:47)

karmaNyEva aDhikArastE mA falEshu kadachan

ma karmafalaheturBhuyam ma te sangostvakarmaNi

Act not from an expectation of the fruit of the action.

Or, in more practical terms, don’t think of the result when performing an experiment!

ACKNOWLEDGEMENTS

I would like to thank my teacher, mentor, role model and friend, Dr.Phalguni Gupta, from the bottom of my heart. He has inspired and supported me through the past 6 years and from him I have learned not just how to perform scientific research, but much more: how to convert challenge to opportunity, how to have an inclusive vision, one that uplifts everybody, how to dream big and make those dreams happen. These are invaluable gifts and I hope I always live by them. His warmth, generosity and nurturing attitude have made the Gupta lab a truly special place.

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1.0 INTRODUCTION

Human Immunodeficiency virus (HIV) is the defining epidemic of our age. In the thirty years since the discovery and description of acute immunodeficiency syndrome (AIDS), its etiological agent, HIV, has become the fastest spreading infectious agent. As of 2009, there are 33.3 million adults and 2.5 million children under the age of 15 infected with HIV in the world. In 2009, an estimated 2.2 million new cases of HIV-1 infection occurred [1]. The economic burden of HIV in the United States alone was estimated in 2006 at $36.4 billion, with $6.7 billion in direct medical costs [2] . Although there is no cure or preventive vaccine for HIV, great strides have been made in our understanding of the virus, its life cycle and its various interactions in the body. This understanding has made the development of antiretroviral drugs targeting key viral proteins possible, thereby helping slow down the progression of the disease, reduce transmission, increase the lifespan of infected individuals and improve their quality of life.

HIV is arguably the most investigated infectious organism. Intense research efforts have helped elucidate not only the virus and its interactions with its host cells, but also have uncovered many secrets of the human immune system, such as non-cytolytic antiviral responses of CD8+ cells, methods of intercellular communication between T cells, B cells and macrophages by the means of nanotubules and exosomes, genetic polymorphisms on MHC molecules that lead to increased or decreased susceptibility to viral infections and so on.

For the rest of this introduction section, we shall take a look at the virus and its life cycle, its effects on various cells of the human body, followed by the different strategies of antiviral defense that are related to the Dissertation objectives .

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1.1 THE HUMAN IMMUNODEFICIENCY VIRUS

The isolation of a retrovirus from a patient with lymphadenopathy at the Pasteur Institute in Paris in 1983 provided the first hint that the causative agent of AIDS might be a retrovirus. Soon afterwards three labs around the world independently isolated the virus and gave it three different names: the virus isolated at Pasteur Institute at Paris was named lymphadenopathy-associated virus (LAV), the virus isolated at University of California at San Francisco was named AIDS-associated retrovirus (ARV), and the virus isolated at the National Institutes of Health at Baltimore was named Human T-cell lymphotropic virus-III (HTLV-III). The name Human Immunodeficiency Virus (HIV) was recommended in 1986 by the International Committee on the Taxonomy of Viruses.

There are two types of HIV: HIV-1 and HIV-2. HIV-2 is associated with lower morbidity and longer incubation periods, although patients with HIV-2 also develop AIDS. HIV-2 is thought to have arisen by interspecies transmission from its counterpart in sooty mangabeys (SIVsmm), while HIV-1 is thought to have arisen from chimpanzees (SIVcpz). HIV-1 strains can be classified into 3 groups: M (for Major), O (for Outlier) and N (non-M, non-O). Group M strains can be further sub-classified into 10 sub-types or clades (A to K).

The genome organization of HIV-1 is summarized in Figure 1. In addition to the three structural proteins present in all retroviruses, Gag, Pol and Env, HIV-1 has 2 regulatory proteins Tat and Rev and 4 accessory proteins, Nef, Vif, Vpr and Vpu (Vpx in HIV-2). The 9 proteins are coded by three, separate open reading frames (ORFs), as shown in Figure 1. The functions of these proteins are given in Table 1.

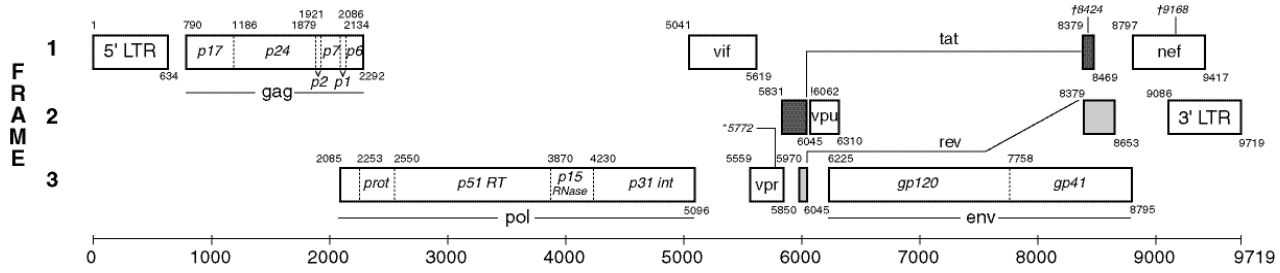


Figure 1: The genome structure of HIV-1
Taken with permission from the Los Alamos Database

Table 1-1: The proteins of HIV-1 and their functions

Proteins of HIV	
Gag	Group Antigen protein is cleaved into 6 smaller proteins, out of which p24 forms the capsid. Other cleaved products form the nucleocapsid (p6, p7), matrix (p17) and spacer peptides p1 and p2.
Pol	Polymerase protein is cleaved to form the reverse transcriptase (RT), protease (PR) and integrase (IN). These proteins are responsible for converting the RNA genome into dsDNA, to cleave Gag, Pol and Env polyproteins and catalyze the integration of the dsDNA into the host genome, respectively.
Env	Env protein consists of 2 subunits: gp120 (also known as Surface or SU subunit) and gp41 (known as transmembrane or TM subunit). These subunits are crucial in mediating the fusion of the virus to the target cell and hence, viral entry into the cell
Tat	Tat is the one of the two regulatory proteins of HIV. Tat (Trans-activator of transcription) interacts with the viral promoter and increasing the processivity of RNAPol II, driving transcription from the HIV LTR promoter.
Rev	Rev (Regulator of virion) protein regulates the export of singly spliced and unspliced viral mRNAs from the nucleus to the cytoplasm.
Nef	Negative Effector protein has many effects, both positive and negative, on viral replication. It decreases the expression of MHC Class I and CD4 molecules to inhibit super-infection; it also increases T cell activation and B cell dysfunctionality.
Vpr	Viral Protein R has a nuclear import sequence, and aids in the entry of the viral pre-integration complex into the nucleus from the cytoplasm. Vpr also affects the cell cycle of the target cell, by causing G2 arrest. Vpr induces neuronal apoptosis
Vpu	Viral Protein U enables viral release from the surface of infected cells by countering the action of the host restriction factor tetherin/BST2
Vif	Viral Infectivity Factor counters the action of the host restriction factor APOBEC3G and retains the integrity of the viral genome, thereby maintaining viral infectivity.

1.2 LIFE CYCLE OF HIV-1

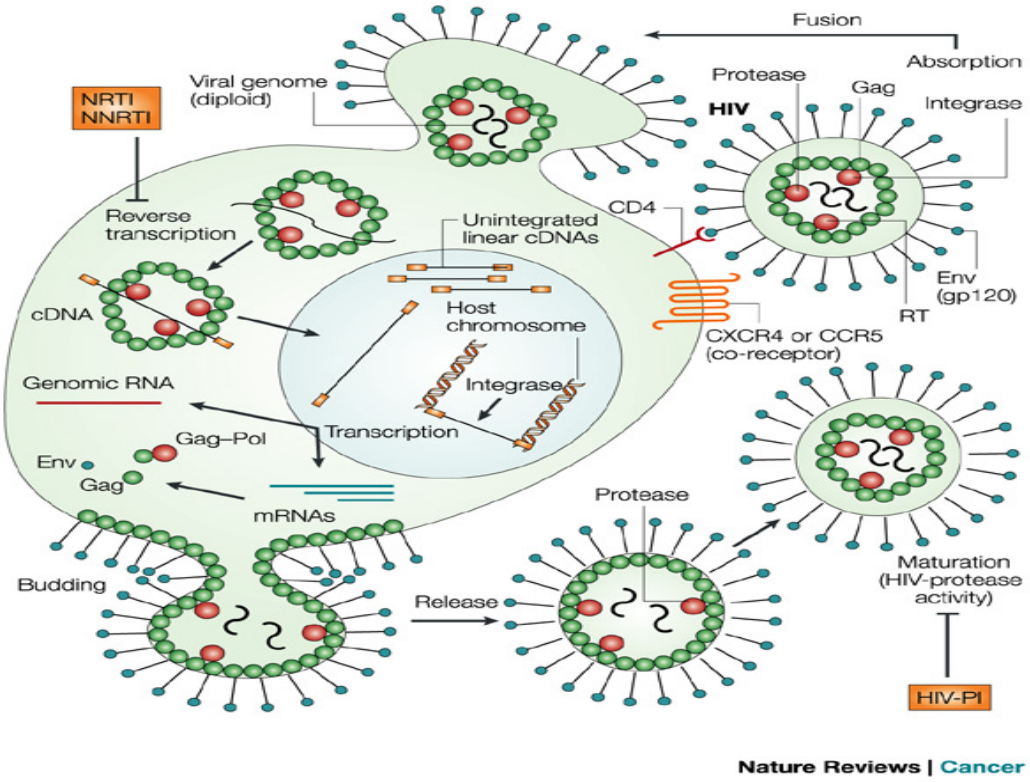


Figure 2: The life cycle of HIV-1

The life cycle of HIV-1: 3 out of the 5 key classes of antiretroviral drugs have been marked. (Taken with permission from Nature Publishing Group). The other main drug classes, not identified in this figure are entry/fusion inhibitors and integrase inhibitors. HIV-1 life cycle begins with entry mediated by gp120 and gp41, followed by uncoating and reverse transcription of the ssRNA genome into dsDNA. The viral protein integrase catalyzes the integration of the dsDNA into the host genome. Efficient transcription and translation enable the production of large numbers of viral proteins and genomes, which are packaged to assemble progeny virions

1.2.1 Viral Entry

HIV requires the protein CD4 as the receptor for its entry. In addition to CD4, the co-receptor proteins, CCR5, primarily found on the surface of macrophages, and CXCR4, found on the surface of $CD4^{+}$T cells, are also required. HIV has long served as the prototype for viruses that infect by fusion at the plasma membrane. The gp120 subunit of the Env protein of the virus

makes contact with the CD4 molecule. The interactions between gp120, CD4 and the co-receptor molecule lead to a conformational change in the gp120. It is thought that these conformational changes on the gp120 expose the inner subunit of Env, gp41. Gp41, a transmembrane protein, embedded in the viral membrane, is also a fusion protein. It is likely that once gp41 is exposed, it inserts into the $CD4^+$ target cell and mediates fusion between the virus and the target cell [3, 4]

Recent evidence shows that HIV-1 can also be endocytosed rapidly, followed by fusion of the viral envelope with the endosomal membrane and mixing of the contents of the virus into the cellular cytoplasm. Thus, HIV appears to be able to use fusion at two sites: at the plasma membrane of the target cell and in the cytoplasm within the endocytotic vesicle [5].

1.2.2 Uncoating and Reverse Transcription

An uncoating event follows fusion, wherein the contents of the viral capsid are released into the cytoplasm. These include the 2 strands of the RNA genome, and the proteins protease, integrase and reverse transcriptase.

The reverse transcriptase (RT) enzyme catalyzes the conversion of ssRNA to dsDNA. This enzyme actually consists of 3 subunits: a reverse transcriptase domain, which converts ssRNA to ssDNA; a polymerase domain, which converts ssDNA to dsDNA; and an RNase H domain, which cleaves and degrades the original RNA template. Fidelity of the reverse transcription process is low, leading to high mutation rates of HIV in vivo. The dsDNA end product of RT, due to the complex process of reverse transcription, contains long terminal repeats at either end, which enable transcriptional regulation upon its integration into the host genome. These long terminal repeats (LTR) are made of three regions: U3, R and U5. Once integration is complete and the retrovirus is in its ‘provirus’ form, the signals present on the LTR

sites lead to complex interactions between various cellular transcription factors, modulating transcription of the viral genome [4].

1.2.3 Integration and Transcription

Integration of the dsDNA product of the RT enzyme is catalyzed by the viral enzyme integrase (IN). Studies show a strong tendency of IN to insert the dsDNA product in sites of the host chromosome that are actively transcribed [6, 7]. Active nuclear import of the pre-integration complex takes place through the action of the viral protein Vpr, and host proteins such as transportin-SR2 and importin 7. Upon nuclear import, the host protein LEDGF/p75 tethers the preintegration complex onto the chromosome [8]. The viral protein IN catalyzes the steps of the DNA integration process and the formation of the provirus.

Transcription from the LTR promoter of the integrated provirus is initially not efficient. The host cell's transcription initiation complex containing RNA Pol II, TATA Binding Protein (TBP) and TBP-Associated Factors (TAFs) assemble at a site known as the Initiator of Small Transcripts (IST), a few basepairs downstream of the actual start site, and initiate transcription. The first few transcripts, though small in length, form the mRNA for the proteins Tat, Rev and Nef. Following protein translation, Tat enters the nucleus, to bind to a bulge-hairpin loop structure formed by the nascent mRNA, known as Transactivating Region or TAR. The binding of Tat to TAR recruits the host transcription factors pTef-b and Cyclin T1. Together this complex of proteins is able to phosphorylate the C terminus of RNA-Pol II, thereby increasing its processivity and enabling the process of transcription elongation. Phosphorylated RNA Pol II can now transcribe full length viral RNA molecules to form both mRNA and the ssRNA genomes of progeny virions. Since mammalian mRNAs are conditioned to be spliced, viral mRNAs that are either only singly

spliced or completely unspliced need to circumvent the splicing mechanism. The viral Rev protein is responsible for this process. It binds an element on the Env mRNA, known as the RRE (Rev Responsive Element) and promotes the egress of unspliced and singly spliced mRNA transcripts, *gag*, *pol* and *env*.

1.2.4 Translation, Protein Synthesis and Egress

Translation of the mRNA transcripts occurs in the cytoplasm. *Gag, pol and env* mRNAs are translated into polyproteins. Gag polyprotein is cleaved into capsid (p24), matrix (p17), nucleocapsid (p7) and p6 proteins, by the viral protease enzyme. Pol is cleaved into RT, protease and integrase enzymes, while Env is separated into gp41 and gp120 subunits. Gag assembly takes place at the plasma membrane. The special affinity for the plasma membrane by Gag is thought to be because of the high levels of the phospholipid PI(4,5)P2 found in this membrane, as opposed to any other cellular membrane [9, 10]. The acyl group of PI(4,5)P2 binds the MA (matrix) protein of the uncleaved Gag polyprotein, thereby anchoring Gag to the plasma membrane and orienting it in the right direction [11]. The viral RNA genomes that are to be packaged within the nucleocapsid are distinguished from viral mRNA by the presence of certain packaging signals, termed ψ(psi), located within the 5' untranslated regions (UTRs) and/or upstream of the coding region of the gag gene [4, 12] . It is thought that the nucleocapsid (NC) on the Gag polyprotein binds to the RNA genomes via the ψ sequences and promotes their encapsidation.

1.3 NATURAL HISTORY OF HIV-1 INFECTION

The course of HIV infection spans three broad stages, based on levels of plasma viremia/ CD4 counts and clinical presentation: the acute stage, the asymptomatic chronic stage, the symptomatic chronic stage and the AIDS stage.

The acute stage of HIV disease, occurring soon after infection, is accompanied by a burst of viral replication and corresponding depletion of $CD4^+$ T cells. Although $CD4^+$ T cell numbers in peripheral blood do not diminish significantly, there is a profound loss of resting memory $CD4^+$ T cells in the gut associated lymphoid tissues (GALT), where the majority of $CD4^+$ T cells reside (Figure 3) [13-15].

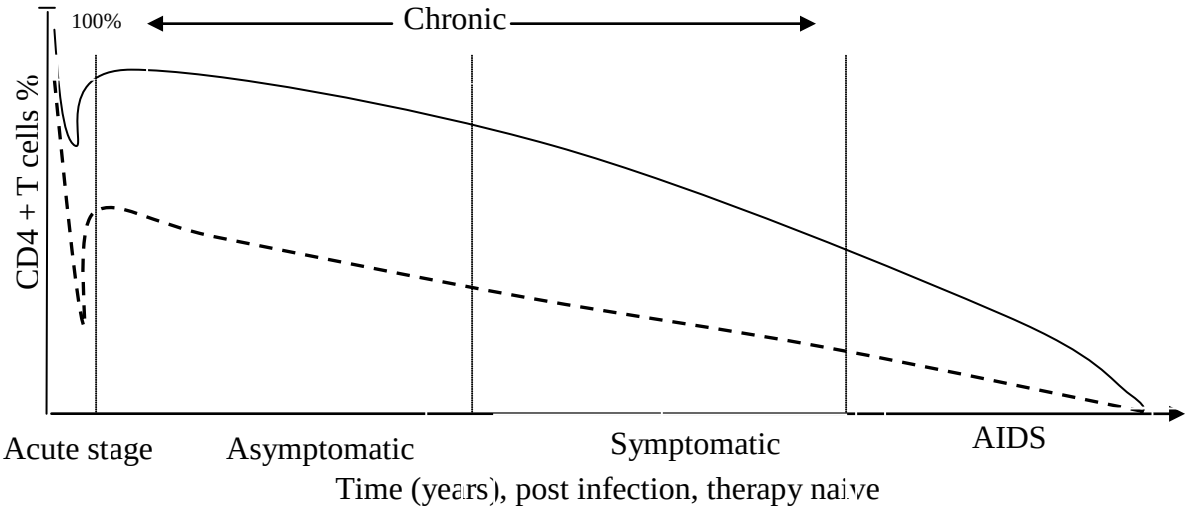


Figure 3: Variation of total and peripheral $CD4^+$T cells with time after HIV-1 infection.

Decrease of total $CD4^+$ T cells (dashed line) and peripheral blood $CD4^+$ T cells (solid line) over time post HIV-1 infection (drawing not to scale).

In SIV models of acute infection in rhesus macaques, depletion of nearly 80% of total $CD4^+$ T cells, by direct killing of the target cells by virus infection, and by virus-induced Fas mediated apoptosis, by 4 days post infection was observed [14, 16]. Thus, the acute phase of HIV infection is characterized by massive virus-induced cell death.

Virus specific $CD8^+$ T cells begin to appear about 3 to 4 weeks post infection (Figure 4). The importance of the CTL response in viral control has been well established with numerous reports showing a temporal association between the emergence of CTL responses and control of virus replication [17, 18], early emergence of CTL escape mutants [19, 20], and the resurgence in viral replication upon the removal of $CD8^+$ T cells [21]. The first $CD8^+$ T cell responses are directed against a narrow breadth of viral epitopes. Although this breadth increases in the chronic stage of the disease, no further viral control can be observed by $CD8^+$ T cells, underscoring the importance and uniqueness of these first responses. Strong $CD8^+$ T cell responses help establish a lower viral set point, thereby improving prognosis for the rate of disease progression [22]. While neutralizing antibodies are produced by B cells a few weeks-months after infection, their role in viral control is still controversial (more on this in later sections). The establishment of a reservoir of latently infected $CD4^+$ T cells also occurs in this stage [23].

The chronic stage of HIV infection is characterized by very different dynamics of viral replication and immune cell turnover, as compared to the acute stage. Outwardly, the chronic stage appears “quiet”: viral loads are stably lower than they were in the acute phase and $CD4^+$ T cell numbers rebound and are stable. However, in actuality, the chronic stage of HIV disease is characterized by a high level of generalized immune activation. Immediately following the loss in $CD4^+$ T cell numbers, a vigorous, self-limiting immune activation is induced to increase the numbers of $CD4^+$ T cells, and correspondingly, the number of target cells for virus infection

[15]. Preferential loss of Th17 $CD4^+$ T cells in the gut leads to increased permeability of the intestines, resulting in microbial translocation from the gut to the peripheral blood, which, in turn, causes systemic activation of immune cells [24, 25]. There have been many lines of research in the recent past to indicate that immune activation is one of the key causes of HIV immunopathogenesis in the chronic stage [26-31]. African Green Monkeys and Sooty Mangabeys, natural hosts of SIV infection and in whom the disease is non-pathogeneic, also experience profound $CD4^+$ T cell loss soon after infection, but do not exhibit the same degree of systemic immune activation soon afterward, as seen in humans or rhesus macaques [27, 30]. Immune activation leads to anergy and apoptosis not only of $CD4^+$ T cells, but also of the other players of the immune system, notably $CD8^+$ T cells and B cells [32-35]. The numbers of $CD8^+$ T cells and B cells are relatively stable through much of the chronic phase of the disease, and begin to reduce around the latter stages of chronic infection, following irreplaceable losses in $CD4^+$ T cell numbers. Dysregulated cytokine production also adversely affects multiple cell types. For example, TRAIL, a member of the TNF superfamily of cytokines, is produced in excess by antigen presenting cells upon HIV infection and leads to apoptosis of $CD8^+$ T cells, monocytes and $CD4^+$ T cells [36, 37].

The stage of AIDS is characterized clinically by $CD4^+$ T cell numbers of $200/mm^3$ or lower. In the absence of effective and appropriate co-stimulatory signals from $CD4^+$ T cells antigen-specific $CD8^+$ T cells undergo activation induced cell death (AICD) upon re-stimulation with antigen, although naïve $CD8^+$ T cells have been noted to be able to respond to antigens [38]. Lack of $CD4^+$ T cell help also leads to anergy in B cells. Opportunistic infections by organisms such as Pneumocystis *carinii*, cytomegalovirus (CMV), HHV8, candida and Cryptococcus are common [39].

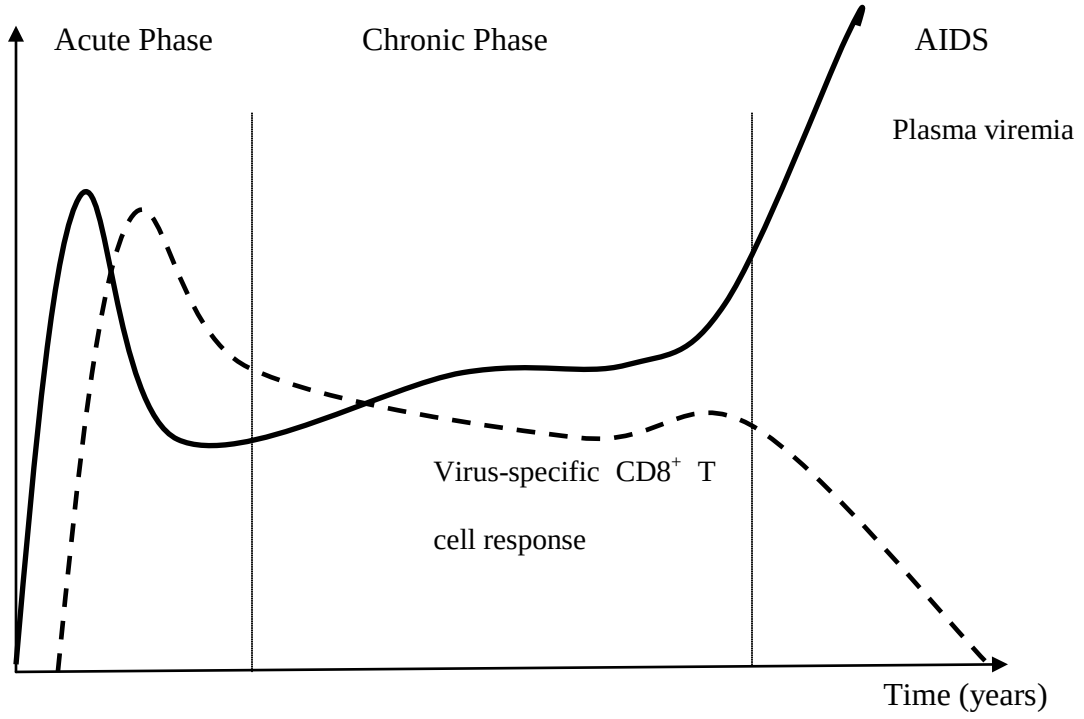


Figure 4: Variation of plasma viremia and virus-specific $CD8^+$ T cell numbers with time after infection with HIV-1.

Variation of virus-specific $CD8^+$ T cells (dashed line) with plasma viremia (solid line) in different stages of HIV disease (drawing not to scale).

1.4 HIV-1 AND THE IMMUNE SYSTEM

There are multiple effects of HIV-1 on the various cells of the immune system, and in turn, there are a variety of ways by which immune cells respond to HIV-1 infection. In this section, we shall take a brief look at the effect of HIV-1 on its major targets, which are the $CD4^+$ T cells, dendritic cells (DCs) and monocytes/macrophages as well as on natural killer (NK) cells, B cells, and $CD8^+$ T cells, as well as the immune responses mounted by each of these cell types to HIV-1 infection.

1.4.1 HIV and $CD4^+$ T cells

$CD4^+$ T cells are one of the two main cell types that are infected by HIV-1. $CD4^+$ T cells also are the major reservoir of HIV in the peripheral blood . HIV-1 infection of $CD4^+$ T cells has many adverse effects not only on the cells themselves, but on the immune system as a whole. Infected cells show disrupted intracellular signaling, do not proliferate as well, are not activated in response to stimuli to the same extent, and cannot interact with antigen presenting cells with the same efficiency. Infected $CD4^+$ T cells also undergo apoptosis faster. HIV-1 infects the various $CD4^+$ T cell subsets, i.e. the naive cells, effector cells, effector memory cells and central memory cells, to different extents. With the infection of the $CD4^+$ T cells, other arms of the immune system are also adversely affected. Lack of $CD4^+$T cell "help" results in dysfunction of $CD8^+$ T cells and B cells. Figure 2 shows the various subsets of $CD4^+$ T cells and is followed by a short summary of the effects of HIV on each of these subsets.

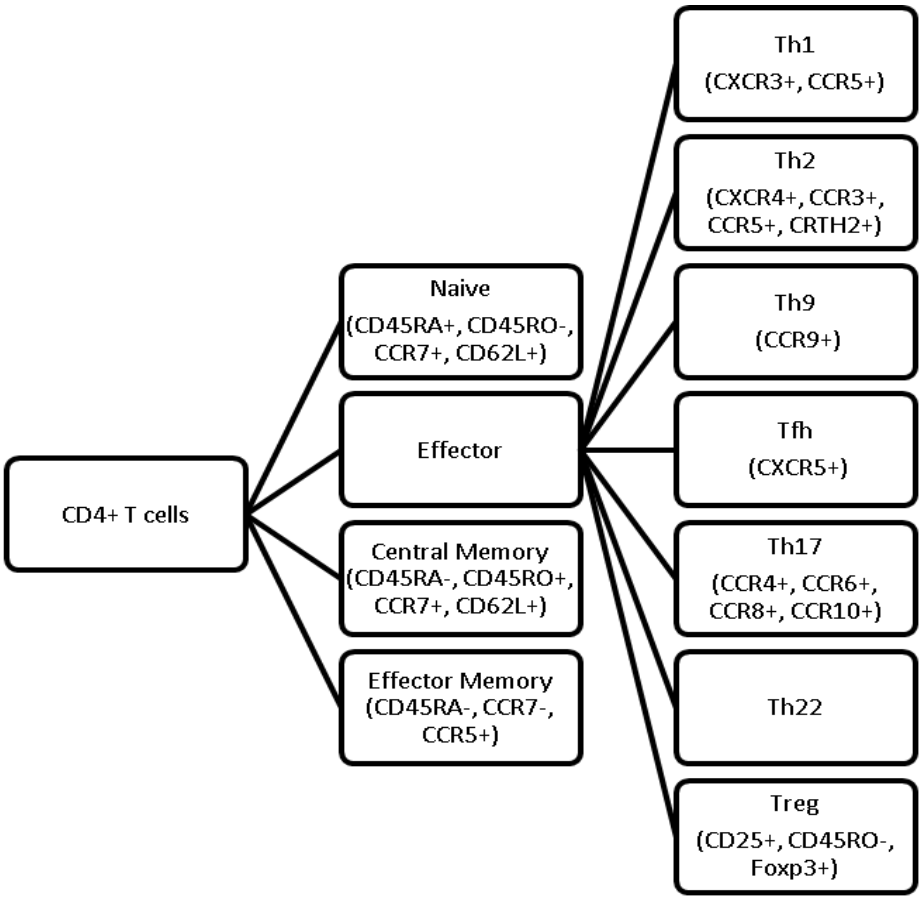


Figure 5: T cell subsets and their molecular markers

Naïve $CD4^+$ T cells: Exposure of naïve T cells to IL4, IL6, IL7 and IL15 in the thymus increases their susceptibility to infection. Although HIV-1 can enter these cells, often, conditions in the naïve cell are refractory to viral replication, with scanty resources, such as a sufficient supply of NTPs for reverse transcription, for crucial steps of the viral life cycle. A low supply of NTPs leads to accumulation of aborted reverse transcripts in the cell, which can cause apoptosis [40]. HIV-1 infection can also disrupts thymic structure, adversely impacting the process of T cell maturation and development [41].

Effector CD4 T cells: HIV preferentially infects HIV-specific $CD4^+$ T cells [42]. Upon HIV infection, activated $CD4^+$ T cells produce the most numbers of infectious progeny virions. HIV infection is associated with apoptosis of all effector T cell subsets. The different mechanisms contributing to this apoptosis include direct cytopathic killing of the infected cell, either by the virus itself or by HIV-specific $CD8^+$ T cells. Secondly, virus infection induces syncytia formation between an infected cell expressing gp120 on its surface and an uninfected target cell expressing $CD4^+$ and CXCR4 or CCR5. The cells thus fused die because of genomic instability. Third, upon productive infection, viral antigens are presented on MHC Class I to $CD8^+$ T cells, causing activation of HIV-specific $CD8^+$ T cells. Activated $CD8^+$ cells cause apoptosis of the infected cells by injection of granzyme B and perforin. Fourth, T cells die by activation- induced-cell-death (AICD). This mechanism serves to limit the immune response by activated effector T cells. However, in HIV infection, there is generalized hyperactivation of T cells, as well as increased resistance of the infected cells to AICD-mediated apoptosis [43]. This results in the death of larger numbers of activated, though uninfected, T cells. Fifth, increased T cell turnover in the presence of constant antigenic stimulation and inflammation may cause T cell anergy. Finally, bystander T cells undergo apoptosis by the action of soluble Tat protein and the resulting enhanced activity of cyclin-dependent kinases [44].

Memory T cells: Memory T cells are preferentially targeted and massively depleted by HIV-1 infection [15]. Effector memory T cells are largely present in mucosal sites, such as the gut and cervix, and are among the first cells to undergo depletion in these sites soon after HIV infection, especially as they express CCR5 protein [45]. Central memory T cells (TCMs) undergo functional impairment upon HIV infection, with decreased cytokine secretion [46], and

decreased ability to differentiate into TEMs [47]. Resting memory cells constitute the main reservoir of latent viruses, leading to viral persistence after therapy.

Regulatory T cells: The effect that HIV and Tregs have on each other is still under study. Tregs could have either a beneficial effect, by reducing T cell hyperactivation and bystander cell apoptosis, or a detrimental effect, by impairing HIV-specific immune responses and maintaining a viral reservoir [48].

Th17 cells: Th17 cells numbers in the gut are significantly reduced after HIV infection. Since Th17 cells are useful in keeping control over bacterial infection, this preferential loss of Th17 cells is thought to cause microbial translocation from the gut [25].

1.4.2 HIV and Dendritic Cells (DCs):

There are at least 2 major subsets of DCs, myeloid (mDC) and plasmacytoid (pDC), classified according to morphology and cytokine secretion [49]. mDCs secrete large quantities of IL12, an interleukin known to be important in the differentiation of T cells and in activation of natural killer cells and T cells [50-53]. pDCs secrete large quantities of IFNα [49, 54], a cytokine known for the antiviral responses in various cells. DCs express several receptors for detecting viruses, such as pattern recognition receptors (PRRs), which include Toll like receptors (TLRs) and C-type lectins [55]. Immature DCs which encounter the virus in peripheral tissues get activated and take up the virus, migrate to draining lymph nodes and trigger adaptive immune responses as well as promoting Natural Killer (NK) cell activation [56]. Activated DCs produce many cytokines, such as IL12, IL15, and IL18. Among these, IL12 is a potent inducer of Th1 responses, which in turn activates the CD8+ cytolytic responses [57]. In addition, recognition of

viral RNA by TLR7 on DCs promotes the secretion of interferon alpha (IFNα). IFNα is known to have both positive and negative effects on the immune system during HIV infection. In the acute phase of HIV infection, IFNα recruits cytolytic NK cells, initiates adaptive immune responses and triggers intracellular antiviral pathways. However, it also acts as a chemotactic attractant to $CD4^+$ T cells, which enter the mucosal sites and present easy targets for viral infection. In the chronic phase, IFNα can cause chronic immune activation of the cells, and also causes apoptosis of infected cells.

HIV affects both mDCs and pDCs in many ways. The number of cells from both subsets of DCs is found to be significantly reduced in HIV positive individuals[58, 59], possibly because of increased apoptosis and/or necrosis of DC subsets upon fusion with HIV [60]. pDCs are also found to secrete lower levels of IFNα [61-63]. There is evidence to suggest that the ability of DCs to stimulate T cells is impaired, possibly because of dysfunctional co-stimulatory molecule expression [64-66]. Immature DCs are thought to have impaired maturation ability in the presence of HIV [67, 68]. A number of reports suggest that DCs transfer HIV-1 to CD4 T cells in lymph nodes, thereby bringing the virus to its target cells. Two types of DC-mediated HIV transmission to $CD4^+$ T cells have been proposed: trans-infection and cis-infection. In trans-infection, HIV-1 virions captured by the DCs are presented to $CD4^+$ T cells across infectious synapses mediated by molecules such as ICAM-1 and DC-SIGN [69, 70]. A second method of trans-infection involves the uptake by $CD4^+$ T cells of DC-secreted exosomes, which enclose HIV virions [71]. Cis-infection of DCs takes place by the productive infection of DCs by HIV-1, either after uptake of free virus, or from infected CD4 T cells, and the formation of progeny virions that can infect $CD4^+$ T cells [72-75].

1.4.3 HIV and Macrophages

Differentiation of monocytes to macrophages is a pre-requisite for the productive infection of these cells by HIV-1 [76-79], as the levels of Cyclin T1 and active pTEF-b are many fold higher in macrophages as compared to monocytes [80]. HIV-1 infection of monocytes and macrophages is characterized by alterations in cytokine production profiles, such as increases in IL1, IL6, IL8, TNF-alpha and other proinflammatory cytokines [81]. The process of phagocytosis is also disrupted by HIV-1 infection. This could be because of the disruption of various signaling process in macrophages [82]. HIV-1 infection of macrophages leads to their apoptosis. Sometimes, however, macrophages can become reservoirs for the virus, providing a protected environment for the latent virus to escape the effects of anti-retroviral therapy. Actively infected macrophages are thought to be the cause for the increasing viremia seen during the stage of AIDS, despite steadily dropping numbers of infected lymphocytes [77].

1.4.4 HIV and Natural Killer (NK) cells

Natural Killer (NK) cells are a key component of the innate immune system and respond to numerous microbes, fungi and parasites. Upon activation, NK cells release cytokines and chemokines that promote inflammatory responses, regulate cell growth, control hematopoiesis and influence the subsequent responses of the adaptive arm of the immune system [83]. One of the common evasion tactics employed by viruses is to down-regulate the expression of the MHC Class I molecule so that viral antigens may not be presented in high frequencies to $CD8^+$ T cells. NK cells counter this action of viruses, by expressing a series of inhibitory NK receptors (iNKRs). iNKRs interact with MHC Class I molecules and inhibit the cytolytic activity of NK

cells. Loss or downregulation of MHC Class I molecules by viruses abolishes this inhibition of NK cells, which proceed to kill the virally infected cell through NK cytotoxicity receptors (NCRs) [84-86].

HIV infection has a number of effects on NK cell activity. The major effects of HIV are on the expression of iNKRs and NCRs, with iNKRs being overexpressed and NCRs being downregulated, by mechanisms as yet unknown [87, 88]. Additionally, NK cells are unable to express their usual complement of cytokines and chemokines, such as IFNγ and TNF-α. They also have a reduced ability to lyse target cells [83]. The HIV proteins Tat, Env and Vpr are known to dysregulate NK cell function in numerous ways. Tat has been shown to inhibit NK cell degranulation and secretion of lytic enzymes by blocking L-type Calcium channels in the cells [89]. Env inhibits NK cell survival and cytotoxicity [90]. Vpr has been shown to dysregulate cytokine signaling by NK cells, by inhibiting the secretion of IL12 and upregulating TGF-β [91], a cytokine that inhibits activation and proliferation of lymphocytes [92].

Recent studies have found an important role for Natural Killer (NK) cells in viral control in the acute phase of HIV infection. While it is not known exactly how NK cells recognize HIV infected cells, different mechanisms have been proposed. NK cells can be activated by interaction with NK activating receptor ligands, such as NKG2D, which is expressed on the surface of infected cells. Infected cells can also present viral epitopes on their MHC Class I molecules, which allows for the activation of Killer cell Immunoglobulin-like Receptors (KIRs), which in turn activate NK cells. Antibodies bound on the surface of infected cells can activate NK cells through interactions with their Fc domains [93]. And finally, downregulation of MHC Class I molecules on infected cells can disrupt the binding of inhibitory KIRs, leading to NK cell activation [55].

1.4.5 HIV and B cells

Recent studies with patient samples at time points immediately before, during and after the peaking of HIV plasma viral load in acute infection have shown that the first detectable B cell response can be seen as early as 8 days after detection of plasma virus. By 13 days post detection of plasma virus, the first HIV-specific antibody could be detected against gp41. Gp120 specific antibodies were detected by 27 days. However, while these first antibodies induced by the virus are capable of binding virions, they have little effect on the timing or magnitude of acute phase viremia [94].

Thus, the first antibody responses are non-neutralizing and directed against gp41 and then gp120. Neutralizing antibodies are seen several months after infection [94-96]. However, the role of broadly neutralizing antibodies in protection is still not determined. Some studies suggest that broadly neutralizing antibodies are not associated with control of viremia [97-99], while others suggest that they are likely to be key components of protection [100-102]. Numerous studies have shown that the development of a broadly protective, neutralizing humoral immune response, in rhesus macaques vaccinated with different strains of SIV, is a complex and lengthy process and can take as long as 6-8 months post infection [103].

A crucial piece of evidence for the importance of humoral responses in protection against acquisition (though not viral control after infection) of HIV comes from the recent ALVAC-prime recombinant Env gp12 boost RV144 phase III trial in Thailand. These results show a 31% vaccine efficacy in preventing acquisition of HIV, and the data implied that this protection is probably mediated by the levels of antibodies elicited early after vaccination [104]. High affinity/avidity antibodies against HIV gp120 are observed to be important in the prevention of vertical transmission of HIV [105].

$CD4^+$ T cell infection by HIV results in profound and extensive immune activation and immaturity of B cells [35, 106]. Patients with HIV frequently are seen to have hypergammagobulinemia and many B cell malignancies [107, 108]. There may be many reasons for this. Activation of B cells could occur by the effects of TNFα, secreted by the infected T cells [109]. The HIV protein, Nef, is also known to stimulate B cells, both through direct interactions and through the induction of the cytokine IL6 in neighboring monocytes [110]. Nef can disrupt IgG2 and IgA class switching in B cells, by entering through nanotubule-like conduits formed by HIV infected macrophages [111]. Although B cells do not get directly infected by HIV, they have been shown to carry the virions on their surface through interactions of gp120 with CD4 and can transmit to T cells making them another reservoir for the virus and another mode of transmission of the virus to $CD4^+$ T cells [112]. There is also evidence to indicate exhaustion and apoptosis of the memory B cell compartment, not restricted only to HIV-specific memory B cells [113].

1.4.6 HIV and $CD8^+$ T cells

$CD8^+$ T cells are important for viral control. It has been observed that there is a temporal association between the emergence of HIV-specific $CD8^+$ responses and a decrease in viral load in the acute phases of the infection [17, 18, 114, 115]. These first $CD8^+$ responses are mounted against a small number of viral epitopes and are able to efficiently suppress viral replication [22]. Individuals who are able to mount strong $CD8^+$ responses in the acute phase have lower viral set points, an accurate predictor of disease prognosis [116, 117]. Thus, $CD8^+$ responses in the initial weeks after infection set the bar for the rate of progression of the disease in the future. As the disease progresses, however, $CD8^+$ T cells responses become more and more impaired due to

increasing dysregulation of the immune system by fast-evolving escape mutants. Even though the breadth and magnitude of $CD8^+$ responses increases in the chronic phase of the infection, there is no further increase in protection or viral control seen [22, 38].

$CD8^+$ T cells, like $CD4^+$ T cells, show chronic hyperactivation upon HIV infection. A majority of $CD8^+$ T cells (80-90% of $CD8^+$ T cells in peripheral blood), not just HIV-specific ones, express activation and proliferation markers, such as CD38 and Ki67, on their surfaces in the acute stages of the disease [32]. This generalized chronic activation and proliferation leads to exhaustion and anergy of $CD8^+$ T cells, which begin to express receptors such as PDL1 (Programmed Death Ligand I), before undergoing apoptosis [33, 34, 118]. $CD8^+$ T cells also require co-stimulatory signals from $CD4^+$ T cells in order to mount efficient responses to chronic HIV infection and to establish memory [119, 120]. In the absence of this help from $CD4^+$T cells, $CD8^+$ T cells are unable to mount secondary responses to already-encountered antigen. They fail to proliferate and undergo activation-induced apoptosis when restimulated via the action of the death receptor TRAIL [121, 122]. $CD8^+$ T cells can continue to secrete cytokines but are impaired in their cytolytic functions, due to inadequate maturation, as measured by expression of CD27 on the surface of these cells, and inadequate expression of perforin [123, 124]. $CD8^+$ memory T cells are also affected by HIV infection. It has been noted that $CD8^+$ memory T cells are predominately in a form that is a precursor to terminally differentiated cells, indicating that the maturation process in these cells is not yet complete. In contrast, CMV-specific memory $CD8^+$ T cells were found to be in the fully mature, terminally differentiated state [125].

The antiviral responses of $CD8^+$ T cells can be classified into cytolytic and non-cytolytic responses. The next sections will discuss each of these categories of responses briefly.

1.4.6.1 Cytolytic $CD8^+$ T cell Responses

Cytolytic $CD8^+$ T cells are, as their name suggests, those that lyse infected cells. $CD8^+$ T cells sample the antigens presented on the HLA or MHC Class I molecules of antigen presenting cells, such as dendritic cells, with their T Cell Receptors (TCRs). The priming of $CD8^+$ T cells by mature dendritic cells requires certain concomitant interactions: the interaction of the TCR with the antigen-loaded MHC Class I molecule on the DC and that of CD28 on the T cell with CD80 or CD86 on the DC, in the presence of key cytokines such as IL12 and Type I interferons. For sustained activation and proliferation of antigen-specific $CD8^+$ T cells, $CD4^+$ T cell "help" is required. Activated $CD8^+$ T cells are loaded with granzymes, granulysin and perforins. When the activated, antigen-specific $CD8^+$ T cell encounters its cognate epitope presented on the MHC Class I molecule of a virally infected cell, perforin is released, which forms pores on the target cell membrane, allowing the entry of granzymes into the cell. Granzymes are serine proteases. Upon entry into the cell, they cleave caspases and activate the caspase cascade, leading to apoptosis of the infected cell.

The role of HLA molecules in cytolytic responses in HIV infection: A clear relationship exists between the HLA Class I alleles, the first $CD8^+$ responses to HIV infection and the progression of the disease. Individuals heterozygous at HLA loci are capable of presenting a broader array of antigenic peptides, resulting in a broader and

more diverse CTL response [126, 127].However, the virus responds by mutating its epitopes so that it is no longer targeted by the HLA antigen presentation system. At a population level, individuals possessing rare HLA alleles are likely to have a selection advantage because the virus develops escape mutations (or other evasion tactics) to evade the most common HLA genotypes [128]. For example, it has been shown that $CD8^+$ cells expressing the allele HLA-Class I B27 or B57 targeted against the p24 gag epitopes KK10 or TW10 respectively, are characterized by poly-functional capabilities, increased clonal turnover and superior functional avidity. These attributes of HLA-B27KK10 and HLA-B57TW10 $CD8^+$ cells are associated with effective control of HIV-1 replication [129]. Such "super-genotypes" include the HLA alleles A1, A2, A3, A24, B7, B27, B58 etc. [128].

1.4.6.2 Non cytolytic $CD8^+$ T cell Responses

In contrast to the classical MHC restricted cytolytic responses, $CD8^+$ T cells also exhibit non-cytolytic responses mediated by secreted factors. Non-cytolytic $CD8^+$ responses were discovered when researchers, seeking a reason for why some HIV infected individuals, known as Long Term Non Progressers (LTNPs), progressed to AIDS at a much slower rate than others, noticed that viral replication in infected cells was lower when the cells were cultured in the presence of $CD8^+$ T cells from LTNPs. Yet, when the $CD8^+$ cells were removed from the culture, viral replication rebounded to its original high levels, indicating that the infected cells had not been killed by the $CD8^+$T cells. Thus, they reasoned, $CD8^+$ cells were able to control viral replication in a non-cytolytic manner [21]. Since then, many non-cytolytic CD8 factors have been described. Among the more well known of these non-cytolytic secreted factors are the entry-inhibiting chemokines RANTES, MIP1alpha, MIP1beta and SDF1 [130]. RANTES, MIP1a and MIP1b

compete for CCR5 and block the binding of R5 viruses to the co-receptor. X4 viruses are blocked by SDF1, which binds CXCR4 [131]. Other $CD8^+$ non-cytolytic factors include ProT-alpha (Prothymosin alpha) and the cytokine IL15, which inhibit HIV transcription in macrophages, but not $CD4^+$ T cells [132] and enhance the non-cytotoxic abilities of $CD8^+$ cells to suppress HIV replication respectively [133].

One of the most important, yet enigmatic, non-cytolytic factors is the CD8 Antivial Factor (CAF). We will now focus on CAF in greater detail.

CAF suppresses transcription from the HIV Long Terminal Repeat (LTR) promoter. Early experiments suggested that suppression of viral replication was highest when there was cell to cell contact between the $CD8^+$ cells and the infected $CD4^+$ cells. However, culture supernatants of $CD8^+$ cells could also suppress viral replication by significant levels [134]. Our lab recently published data showing that exosomes, which are 100nm vesicles secreted by cells, from $CD8^+$ cells could also efficiently suppress viral replication [135]. Thus, it was inferred that CAF was present not only on the cell membrane of $CD8^+$ T cells, but could also be secreted in the form of exosomes and might possibly even be cleaved off the surface of exosomes to be secreted into the supernatant. It can be inferred from its soluble nature that this factor does not require MHC restriction to function as an anti-HIV molecule, and indeed, experiments have proved its action in the absence of HLA compatibility [136]. The identity of CAF is still unknown. It has been shown by many groups that CAF does not identify with the cytokines or chemokines listed in Table 1-2 [130, 132, 137-147].

Table 1-2: List of chemokines and cytokines that do not identify with CAF

TNF-alpha, TNF-beta, IFN alpha, IFN beta, IFN gamma,
TGF beta,
GMCSF, GCSF, RANTES, MIP-1alpha, MIP-1beta,
Monocyte chemoattractant protein MCP-1, MCP-2, MCP-3,
SDF-1,
Growth regulated oncogene GRO-alpha and GRO beta,
Lymphocyte Inhibitory Factor LIF-1,
Interferon Regulated Protein IP-10,
Granzymes A and B, Granulysin, Lymphotactin,
alpha-defensins,
Natural Killer cell enhancing factors (NKEF),
Rnase,
Protegrin,
Histanins,
Prothymosin alpha

The CAF-mediated inhibition of viral replication is found to correlate with the stage of the disease, and is usually most potent in the acute stages of the disease and may contribute to the establishment of the viral setpoint [148-151]. It is also observed in exposed-but-uninfected individuals and Long Term Non-Progressers, suggesting its importance in viral control [21, 147,

152]. CAF is also increased following antiretroviral therapy. CAF is equally effective against various tropisms and clades of HIV-1, HIV-2, SIV, and FIV [21, 153-159]. The $CD8^+$ cells that secrete CAF have not been found to be HIV-specific, and $CD8^+$ cells from patients with CMV and EBV have been found to be equally effective. Characterization of the cells that produce CAF show that these cells have the activation markers HLA-DR and CD38 [160, 161].

The mechanism of action of CAF is also unknown. Some years ago, it was shown that culture supernatant from transformed $CD8^+$ T cells could be used as a source of CAF, instead of obtaining primary $CD8^+$ T cells and culturing them [162-164]. Using transformed $CD8^+$ T cells and their culture media as a source of CAF, it was shown that the signaling molecule STAT1 was necessary for the process of suppression [165]. However, information about upstream and downstream interacting partners is as yet unknown, as is the exact mechanism of transcription suppression.

Thus, we sought to determine the mechanism of action of CAF.

2.0 OVERALL AIM AND SPECIFIC AIMS

Overall aim: Our overall aim is to elucidate the mechanism of action of the $CD8^+$ Antiviral Factor, which leads to the transcriptional suppression of HIV-1 Long Terminal Repeat promoter.

Rationale and Hypothesis: Our preliminary studies on the kinetics of CAF action showed that target cells needed ~12-16 hour interval after the addition of CAF to provide maximum transcriptional suppression of HIV-1 LTR. This indicates a need for intracellular signaling and possibly production of an intermediate protein. Furthermore, we and others have shown that CAF action is mediated via the signaling protein STAT1. Since CAF has been shown to suppress gene expression from the viral promoter, , there may be one or more regions on the viral promoter that experience changes in protein binding, upon CAF addition which may ultimately lead to a reduction in transcription from the promoter.

Therefore, we hypothesized that CAF triggers a signal cascade upon its interaction with target cells. This cascade of signals ultimately converges upon the integrated promoter of HIV-1, the Long Terminal Repeat (LTR), and causes changes in protein binding, which lead to suppression of transcription from the promoter.

2.1 SPECIFIC AIMS

1. To study the interaction between CAF and its target cells

2. To determine the intracellular signal cascade that is triggered in response to CAF that leads to transcription suppression.

3. To determine the region on the LTR promoter that is crucial for the CAF mediated transcriptional suppression.

3.0 MATERIALS AND METHODS

Primary cells, cell lines and virus stocks: Primary $CD8^+$ T cells were isolated from the blood of nine therapy-naïve HIV-1 infected subjects, with CD4 counts greater than 400/uL from the Multicenter AIDS Cohort Study (MACS) with Institutional Review Board approval. CD8+ T cells were isolated using Dynal ™ immune-magnetic beads coated with antibody to CD8 protein, according to manufacturer's instructions (Life Sciences Technologies, Carlsbad, CA). Isolated $CD8^+$ T cells were grown in RPMI supplemented with 20% FBS, rIL2 (50U/mL, Roche Diagnostics), 25mM HEPES and the antibiotics Penicillin (100U/mL) and Streptomycin (100ug/mL) for 14 days. On day 14, cells were centrifuged at 300g and the resulting supernatant was used for further investigations. Primary CD8-negative T cells were prepared by immune-magnetic depletion of $CD8^+$ T cells from peripheral blood mononuclear cells (PBMCs) of uninfected subjects, as previously described [166]. CD8- negative PBMC were grown in the presence of phytohemaglutinnin for 3 days to activate and expand the $CD4^+$ T cell population, followed by infection with the R5 primary viral isolate HIV_{015} as previously described [166]. The $CD8^+$ T cell line, TG, was previously established by Herpes virus saimiri (HVS)-transformation of $CD8^+$ T cells from a chronically HIV-1 infected subjected from the MACS [167]. TZMbl cells were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID from John C Kappes, Xiaoyun Wu and Transzyme Inc. HeLa-Tat cells were also obtained from the NIH AIDS Research and Reference Reagent

Program from William Haseltine and Ernest Terwilliger. The fibrosarcoma cell lines U1A, U2A, U3A, U4A, U6A, U3AR, G1A and G2A were a kind gift from Dr.George Stark (Cleveland Clinic, Cleveland, Ohio). 293T cells were obtained from the ATCC. TZMbl cells were grown in RPMI supplemented with 10% FBS and 25mM HEPES. The fibrosarcoma cell lines and 293T cells were cultured in DMEM, supplemented with 10% FBS, 2mM Glutamine and 1mM sodium pyruvate. The R5 primary viral isolate HIV-1 015 was obtained from the PBMC of HIV-1 infected subjects enrolled in the MACS of University of Pittsburgh, as previously described [166, 167].

Plasmid constructs: Full length HIV LTR-CAT and the successive deletion constructs at EcoRV, BstNI, HaeIII and MnlI have been described in [168] and were a gift from Dr.Mario Estable of Ryerson University, Toronto, Canada. Wt-LTR-luciferase was created by amplifying the full length LTR with the primers ACCTCGAGTTAGTCAGCCATGTC (Fwd) and GTGAAGCTTTTCCTTAGCTCCTGAA (Rev), digesting the product with XhoI and HindIII and ligating it into the pGL4.10 (Promega, Madison, WI) cut with the same enzymes. The BstdelTAR construct was made by digesting the BstNI construct with KpnI and BglII, blunting the ends with Klenow fragment and then ligating the product with T4 DNA ligase. SpI deletion mutants were created by overlapping PCR, using the specific DNA oligonucleotides and the corresponding primers listed in the Appendix section. For the point mutations, the mutations introduced to inactivate the SpI sites (underlined) are marked in bold, where G nucleotides were substituted by T: **TT**CGTGGCCTG**TT**CGGGACTGG**TT**AGTGGC. The oligonucleotide pairs and the corresponding primers used to create these constructs are also listed in the Appendix section. For the deletion constructs, each pair of oligonucleotides was added, instead of template DNA, to the PCR mix and the reaction was cycled without primers for 5 times, with these

conditions: (94C, 2’30”; [94C-1’, 55C-1’, 72C- 1’45”]X5; 72C-10’), the primers were then added at a final concentration of 10uM and the tube was cycled for 25 cycles at the same cycling temperatures and times. The PCR product was purified, digested with HindIII and SacI and ligated, using T4 DNA ligase, to the BstdelTAR construct cut with the same enzymes. For the point mutations, each of the forward probes (K- P) was added along with Oligo 2, at a final concentration of 10mM and the corresponding reverse primer, at a final concentration of 1uM and PCR amplified for 10 cycles at the following conditions: (94C, 2’30”; [94C-1’, 55C-1’, 72C-1’45”]X10; 72C-10’). Primer a fwd was added at a final concentration of 1uM, and the reaction mixture was cycled 25 more times at the same conditions. The PCR product was purified, digested with HindIII and SacI and ligated, using T4 DNA ligase, to the BstdelTAR construct cut with the same enzymes. The sequences of all constructs were confirmed by sequencing with the primer 5’CGCTGGGCCCTTCTTAA, present on the luciferase gene.

Antibodies and other reagents: Antibody to pSTAT1 (Y701 site) was obtained from Millipore (Billerica, MA). Antibodies to pSTAT3 and pSTAT5 (clone C11C5) were obtained from Cell Signaling Technology (Beverly, MA).

Purification of exosomes. Exosomes were harvested from culture supernatants by adaptation of previously described methods involving serial centrifugation of the culture supernatant, followed by sucrose density gradient purification (18, 37). Culture fluid from TG cells or primary $CD8^+$ T cells from HIV-1-infected subjects was harvested by centrifugation at 300g for 10 min to deplete cells. Cell-free culture supernatant was subjected to serial centrifugations of increasing centrifugal force to derive supernatants and pellets at 800g for 30 min, 6,000g for 30 min, 15,000g for 30 min, and 60,000g for 1 h, with all spins performed at 4°C. The 15,000g pellet was then resuspended in HBSS and subjected to a discontinuous sucrose

density gradient centrifugation at 90,000g at 4°C through a 40% sucrose (1.14 g/ml) layer overlaid over a 60% sucrose (1.21 g/ml) cushion. After centrifugation, the membrane fractions banded over the 40% and 60% sucrose interfaces were removed, diluted in HBSS, and re-centrifuged at 18,000g to purify the fractions. The membrane pellet was resuspended in HBSS and analyzed for the presence of exosomes. Exosome protein concentration was measured using a Bradford assay (Bio-Rad, Hercules,CA).

Quantitative acute HIV-1 replication suppression assay: A quantitative acute HIV-1 replication suppression assay was performed as previous described [166]. Briefly, $CD4^+$ T cells acutely infected with HIV 015 were incubated in the presence of CAF for 5 days. On day 5, the extent of productive HIV-1 replication was assayed by measuring the concentration of extracellular p24 in culture supernatant using a standardized p24 ELISA kit (Perkin Elmer, San Jose, CA). MTT assays were also performed to measure cell viability, as previously described [169].

Quantitative LTR promoter suppression assay: Assays of HIV-1 transcription suppression using the TZM-bl cell line were performed as previously described [135]. For all other target cell lines, HIV-1 LTR transcription suppression was assessed by transfection of the Wt-LTR-luc plasmid. Cells to be transfected were plated in antibiotic fee medium in 96 well plates at a concentration of 20,000 cells/well. Twelve hours after plating, CAF was added at either 10% vol/vol or 50% vol/vol concentrations depending on whether the source of CAF was transformed TG cells or primary $CD8^+$ T cells from HIV-1 infected patients, respectively. Twenty four hours after the addition of CAF, cells were transfected with 1nng of Wt-LTR luc plasmid per well, using Lipofectamine LTX and Plus reagent (Life Sciences Technologies, Carlsbad, CA), according to manufacturer's instructions. Twenty four hours after transfection,

cells were stimulated with 100ng/mL phorbolmyristoylacetate (PMA) for six hours and were assayed for both luciferase production by Bright Glo Luciferase Assay System (Promega, Madison, WI) according to manufacturer's instructions, and cell viability by MTT assay, previously described [169].

Confocal microscopy of exosome-cell interaction. TZM-bl or HeLa Tat cells were labeled, according to the manufacturer's instructions, with PKH-67 (Sigma, Dekalb, MO), a lipophilic dye that binds cell membrane lipids by its long aliphatic tail. Cells were plated on 35-mm uncoated dishes (type P35G-0-10C; Mattek Corp., Ashland, MA) at 75% confluence in 10% RPMI medium. Freshly harvested exosomes were quantified for protein concentration by Bradford's assay (Bio-Rad Laboratories). Exosomes were then resuspended in HBSS to a total volume of 1 ml. A 1:250 dilution of the Cy5 dye (GE LifeSciences, Buckinghamshire, United Kingdom) was made, and 10 µl of the diluted dye was added to 30 _g of exosome protein. The incubation of dye with exosomes was performed at 4°C in the dark for 1 h. Following incubation, the exosomes were washed three times with HBSS and resuspended to a concentration of 1 to 1.5 µg/µl. Thirty micrograms of labeled exosomes was added to each dish of labeled cells, and incubation was carried out for 10 min or for 12 h at 37°C in the dark. Fixation was done at the required time points by 2% paraformaldehyde. Dishes containing fixed cells and exosomes were viewed under an Olympus Fluroview 1000 instrument. Images were analyzed using MetaMorph software.

Depletion of Interferon gamma (IFNγ) and IFNγ ELISA: IFNγ ELISA kits were obtained from eBioscience (San Diego, CA). Samples to be depleted of IFNγ underwent three successive incubations on IFNγ antibody coated plates, beginning with an overnight incubation at 4C. Samples were removed, placed in new antibody-coated wells, and incubated for 2 hours at

room temperature. They were once again placed in new antibody-coated wells for a final overnight incubation at 4C, before being removed and evaluated for residual IFNγ by ELISA, according to manufacturer's instructions. Control samples underwent the same incubations on rabbit IgG antibody-coated plates.

Protein phosphorylation measurements: For time dependence experiments, CAF from TG cells was added at a 10% vol/vol concentration to 10^6 cells/mL of either TZMbl cells or acutely HIV-1 infected $CD4^+$ T cells for different periods of time ranging from 1 minute to 24 hours before cell lysis. For dose dependence experiments, CAF from TG cells was added, at concentrations ranging from 0.05% vol/vol to 15% vol/vol to 10^6 cells/mL of either TZMbl cells or acutely HIV-1 infected CD4+ T cells for 15 minutes before cell lysis. Cell lysates were aliquoted and stored in the presence of protease inhibitors until further use. Quantification of total and phosphorylated STAT1 was performed by Luminex ™ Map platform (Luminex, Austin, TX), using Mapmates ™ reagents (Millipore, Billerica, MA). Phosphorylation of the proteins Crk, FRS2, PLC-g2, Jak3, STAT3, p38 MAPK, JNK, Tec kinase, PI3K and IL15Ra was quantified using Epiquant ™ system (Millipore, Billerica, MA). Briefly, acutely HIV-1 infected CD4+ T cells, at a concentration of 10^6 cells/mL, were treated with 50% vol/vol IFNγ depleted or control CAF samples from primary CD8+ T cell culture supernatants for 10 min, 20 min, 12 hours or 42 hours. Control cells treated with media only were included for each time point. cells were lysed, processed and digested according to the kit's protocol. Digested lysates were then incubated with antibodies against the phosphorylated peptide-forms of the above mentioned proteins and analyzed by Luminex 1000 instrument. The phosphorylation of each protein was compared to that found in the media control and the fold change from the media control was calculated.

CAT and luciferase assays: 293T cells were plated at a concentration of 20,000 cells/100uL in a 96 well plate. 24 hours after plating, 10% vol/vol of CAF from TG cells was added to the culture. Following an additional 24 hours, 1ng of the relevant plasmid was transfected into all cells, using Lipofectamine Plus (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. 24 hours following transfection, the cells were stimulated with 100ng/mL phorbolmyristoylacetate (PMA) for 6 hours, after which they were lysed and the amount of CAT protein or luciferase was measured. CAT and luciferase protein expressions were quantified by CAT ELISA (Roche) and Bright Glo expression systems (Promega), respectively, according to manufacturer's instructions.

Western blots: Cell lysates were run on 10% SDS protein gels (Pierce Proteins, Rockford, IL), transferred on PVDF membranes (Millipore, Billerica, MA), blocked for 1 hour using 5% non fat milk and then probed for the expression of phosphorylated STAT1, STAT3 and STAT5. The secondary antibody used was goat anti rabbit antibody conjugated to horse radish peroxidase

4.0 STUDIES ON THE INTERACTIONS BETWEEN THE CD8 ANTIVIRAL FACTOR AND TARGET CELLS

4.1 ABSTRACT

The CD8 Antiviral Factor (CAF) is a $CD8^+$ T cell-secreted factor that is known to suppress HIV-1 transcription. The identity and mechanism of action of CAF are unknown. We found that small, membrane-bound vesicles, named exosomes, secreted by $CD8^+$ T cells contained high levels of CAF and were able to potently suppress HIV-1 transcription. We used these exosomes as a source of CAF to examine the interactions of CAF with its target cells. We observed that CAF-containing exosomes interacted with target cells at their surface within 10 minutes of addition. However, maximal suppression of transcription was seen in these cells only 12 to 16 hours after addition. Exosomes could also be washed away within 5 minutes of addition to cells and would still produce significant suppression of LTR driven transcription, after a 12 to 16 hour incubation of the cells. These results indicate that HIV-1 transcriptional repression is triggered by one or more ligand-receptor interactions that occur upon membrane contact of exosomes with the target cells and the possible involvement of an intracellular secondary gene expression, Furthermore, exosome-mediated transcriptional suppression is dependent on STAT1 signaling pathway.

4.2 INTRODUCTION

Previous studies on the nature of CAF found a dual presence of CAF, on the CD8 membrane, as well as culture supernatant. Although cell-to-cell contact between the $CD8^+$ T cell and the target $CD4^+$ cell resulted in maximal HIV-1 transcriptional suppression, CD8 culture supernatant could also suppress HIV-1 transcription efficiently [134]. Studies on the requirements of MHC compatibility between the $CD8^+$ T cell and $CD4^+$ target cell for CAF activity showed that CAF could act even when there was no MHC compatibility between the $CD8^+$ T cells and the infected $CD4^+$ T cells [136]. Thus, it appeared that CAF could be found in a membrane-associated form, on the $CD8^+$ T cell surface, as well as in a soluble form in cell culture supernatant.

One mechanism by which this could occur is by proteolytic cleavage of a membrane bound factor and release of the active form into the extracellular medium. However, experiments to test this proved inconclusive [170]. Another mechanism by which a surface bound protein might be secreted into the extracellular environment is through the process of endosomal trafficking. Cell surface proteins are continuously endocytosed by the cell into early endosomes. Early endosomes have many cellular fates [171, 172]. One possibility is that their contents might be degraded, following fusion of the endosomes to lysosomes. A second possibility is that the contents of the early endosomes may be recycled back to the surface [171]. A third possibility involves the fusion of many early endosomes into multi-vesicular bodies which then traffic back to the cell surface, and release the individual endosomes into the extracellular medium, in the form of “exosomes” [173, 174]. If CAF were indeed trafficked through endosomes from the cell surface, and secreted into the extracellular medium in the form of exosomes, it would not only still be membrane-bound, but it would also be present in the culture supernatant of $CD8^+$ T cells,

thereby reconciling the various pieces of data showing that it were both membrane bound as well as soluble.

Exosomes are ~100nm-sized spherical, membrane-bound bodies secreted by cells either constitutively or upon activation. They are formed by the aggregation of endosomes inside multi-vesicular bodies (MVBs). While most MVBs are degraded after fusion with lysosomes, some MVBs are recycled back to the cell surface and the endosomes contained within them are released in the form of small vesicles, known as exosomes. Exosomes are usually range from 1.1-1.9g/cm3 in density. The protein composition of exosomes has been seen to be cell type specific, although some proteins, such as CD63, CD81 and other members of the tetraspanin family, are found on all exosomes, regardless of the origin of the secreting cell [175]. Exosomes can be isolated from the culture supernatant of cells by a series of centrifugation steps, followed by density gradient purification. Our studies showed that CAF was present on the surface of exosomes and its suppressing activity could not be abolished even after the removal of peripheral proteins on the exosomes [135]. Thus, it appeared that the CAF protein complex was present as an integral membrane protein on the exosome surface.

Since the identity of CAF is unknown, it was impossible to study its interactions with target cells. We therefore investigated transcriptional suppression by exosomes that carried CAF and tracked their interactions with target cells. Confocal microscopy indicated that interaction of exosomes with $CD4^+$ target cells was limited to the cell surface, with no evidence for their internalization. Furthermore, our data indicate that an intracellular signaling mechanism is involved in exosome-mediated suppression of HIV-1 transcription.

4.3 RESULTS

1. Exosomes from $CD8^+$ T cells are able to suppress HIV-1 LTR driven transcription: It is known that $CD8^+$ T cells and culture supernatant of these cells can exhibit CAF-mediated suppression of LTR-driven gene expression. Previous studies from our lab showed that membranes of transformed $CD8^+$ T cells could suppress HIV-1 replication in infected primary $CD4^+$ T cells by >90% [135]. We noticed that, in addition to the cell membrane, the fraction of the culture supernatant containing components of density between 1.14-1.2g/cm3, could also suppress HIV-1 replication to nearly 100% (Figure 6A). This component contained 100nm-sized vesicles, known as exosomes (Figure 6B)

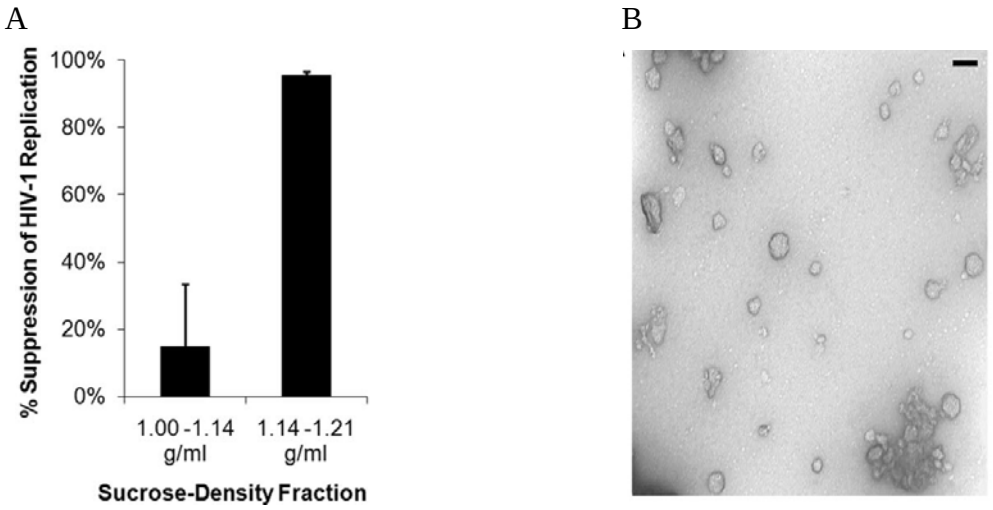


Figure 6: Suppression of HIV-1 replication by CD8+ T cell culture supernatant fractions

Culture supernatant from a transformed CD8 T cell line was serially centrifuged at 300g, 600g, 6000g and 15,000g. the 15,000g fraction was then purified on a sucrose density gradient. The fractions corresponding to 1-1.14g/cm3 and 1.14-1.21g/cm3 were isolated, purified and assayed for HIV-1 replication suppressive activity (A). The fraction corresponding to 1.14-1.21 g/cm3 was observed under electron micrograph after purification (B). Figure used with permission from the Journal of Virology

We isolated these exosomes and characterized the proteins present in them. We found that although the exosomes were derived from $CD8^+$ T cells, they did not express the CD8 molecule on their surface (Figure 7A), in contrast to the CD8+ T cell membrane (Figure 7B). However, CD63, a late endosome/lysosome marker, was readily detected on the exosome surface, indicating its origins in the endosomal compartment. Other tetraspanins, such as CD9 and CD81, were also enriched in the exosomes, as was the antigen presenting molecule, MHC Class II.

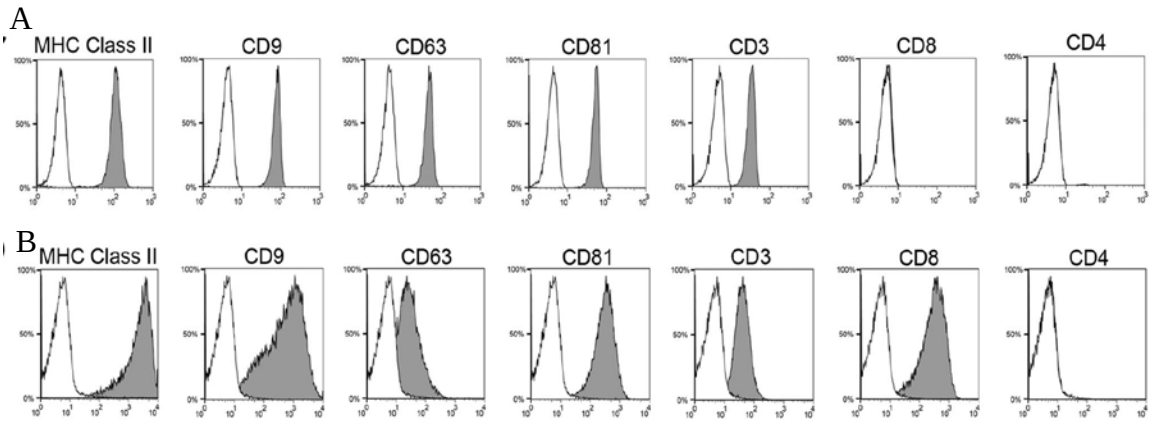


Figure 7: Flow cytometry analysis of proteins on the exosome and CD8+ T cell membrane surface

Flow cytometry analysis was performed on exosome-coated beads (A) and transformed CD8 T cells (B) Unshaded areas represent the antibody isotype; shaded areas indicate specific fluorescence by the antibody labeled above each graph. Figure used with permission from the Journal of Virology [135]

We determined whether exosomes secreted by these cells could also potently suppress LTR transcription. TZM-bl cells were exposed to $CD8^+$ T cell-secreted exosomes for 24 hours, after which they were infected with HIV-1 virus to stimulate gene expression of the reporter gene beta-galactosidase. We found that mRNA levels of beta-galactosidase

were decreased when cells were treated with CD8 exosomes, compared to control (Figure 8). This indicated that CAF on exosomes secreted by $CD8^+$ T cells could potently suppress HIV-1 LTR driven reporter gene transcription.

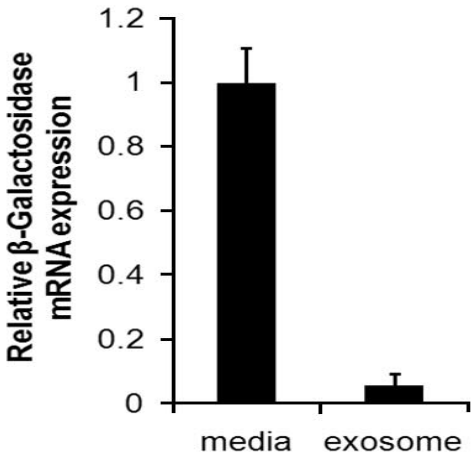


Figure 8: $CD8^+$ T cell derived exosomes suppress transcription from the HIV-1 LTR.

Real-time RT-PCR measurement of LTR-activated β-galactosidase mRNA in TZM-bl cells in the absence or presence of exosomes. Values for β-galactosidase mRNA measurements were normalized by co-quantification of β2 microglobulin rRNA. Figure used with permission from the Journal of Virology [135]

2. <u>The suppression of LTR gene expression by exosomes from $CD8^+$ T cells is time dependent</u>: We next sought to determine if suppression of LTR-driven beta galactosidase expression by exosomes was dependent upon the duration of interaction between the cells and the exosomes. $CD8^+$ T cell-derived exosomes were added to TZM-bl cells for various periods of time from 1 hour to 16 hours, following which cells were infected with HIV-1 to stimulate HIV-1 LTR driven gene expression. The suppression of beta galactosidase was calculated in cells that received the exosome treatment, relative to control cells. We found that there was a high correlation between the duration of the exosome treatment

and the degree of suppression of LTR gene expression. We also determined that maximal suppression (>85%) was found only after 12-16 hours of incubation post addition of exosomes to the cells (Figure 9).

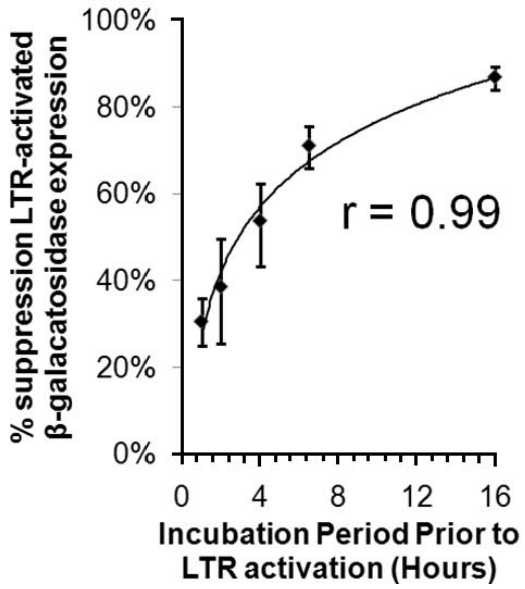


Figure 9: Time dependent suppression of LTR-driven gene expression by $CD8^+$ T cell derived exosomes

Percentage suppression of LTR-driven beta galactosidase production in TZM-bl cells treated with CAF-exosomes for increasing periods of time, n=3. Suppression was calculated by comparison with untreated controls. Figure used with permission from the Journal of Virology, [135]

3. Suppression by exosomes does not require the continued presence of exosomes: As shown above, a 12-16 hour time interval was required by cells post addition of exosomes to exhibit potent suppression of LTR driven transcription. We considered the possibility that this time interval could be due to the activation of some signaling pathways within the target cell in response to CAF, which might ultimately lead to transcription suppression of the LTR. Thus, if CAF was triggering some kind of signal response,

continued presence of the CAF-containing exosomes probably would not be required for maximal suppression to be seen 12-16 hours later. To test this possibility, we washed away CAF-containing exosomes at different time points after addition to TZM-bl cells, and let the cells incubate in the absence of exosomes for additional 12-16 hours and then challenged with HIV-1. Subsequently, we measured suppression of beta galactosidase expression. As shown in Figure 10, the level of gene expression in cells exposed to exosomes for 5 minutes are comparable to cells exposed to exosomes for 16 hr. These results support the idea that CAF mediated suppression of HIV-1 may require synthesis of a secondary signal (protein) which may be ultimately responsible for transcriptional suppression of HIV-1

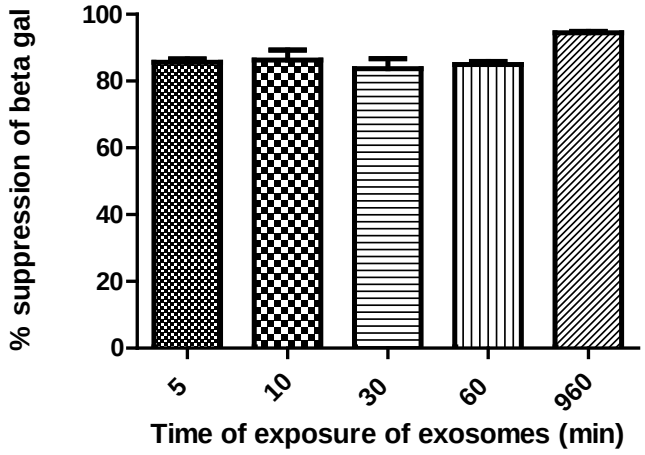


Figure 10: Continuous exposure of target cells to exosomes is not necessary for maximal suppression

Suppression of LTR-driven beta galactosidase expression in TZM-bl cells treated with CAF-containing $CD8^+$ T cell exosomes for various periods of time ranging from 5 minutes to 960 minutes and then washed to remove the exosomes.

4. Interaction of $CD8^+$ T cell exosomes with target $CD4^+$ cells: The presence of an HIV-1 suppression factor on the extracellular surface of the exosomes as well as a time-dependent induction for LTR promoter repression suggested the involvement of a CAF-triggered signaling mechanism. We therefore sought to determine whether the exosomes directly interacted with the surface of target cells in their induction of HIV-1 suppression. We performed confocal microscopy using TZM-bl cells labeled with the lipophilic dye PKH67 to stain internal and external cell membranes. Exosomes were labeled with Cy5, a dye that covalently binds to the primary amine group of proteins. The Cy5-labeled exosomes were incubated with PKH67-labeled cells at 37°C in the dark for various times, and three-dimensional confocal microscopy was performed following fixation. After only 10 min of coincubation, exosomes were found to concentrate at the cell surface, limiting their localization only to the periphery of TZM-bl cells (Figure 11, left panel). After 12 h of co-incubation, a time by which near-maximal HIV-suppressive activity was detected (Figure 9), the Cy5-labeled exosomes were still limited to the periphery of TZM-bl cells (Figure 11, right panel). No evidence for internalization of exosome membrane was found at 12 hr. This seemed to imply that the exosomes do not enter the cells, and the CAF on the exosomes interacts with the target cells at their surface.

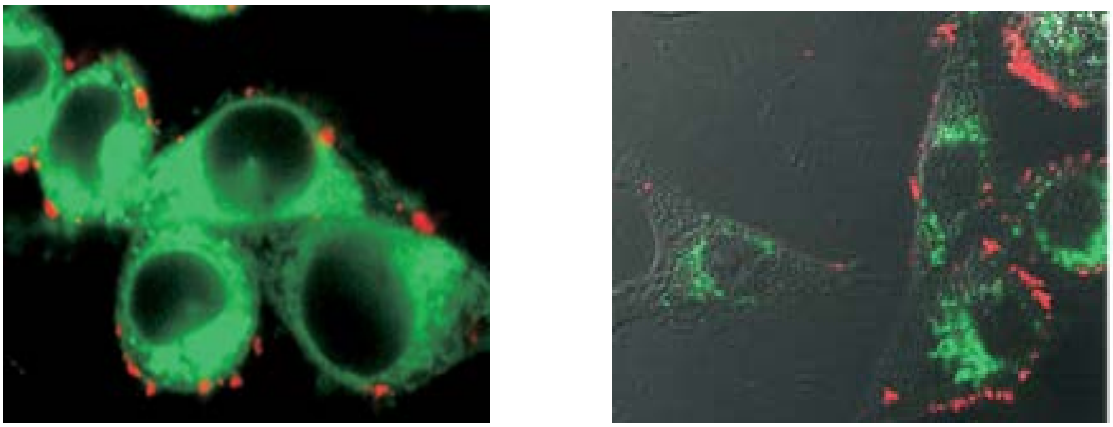


Figure 11: Interaction between exosomes and target cells is restricted to the cell surface

Interaction between Cy5 labeled (red) exosomes on the surface of TZM-bl (green) cells at 10 minutes (left panel) and 12 hours (right panel) post addition of exosomes to cells. Images were taken by Olympus 1000 Floview microscope, after fixing the cell. Figure used with permission from the Journal of Virology, [135]

5. CAF from $CD8^+$ T cell derived exosome signals via STAT1: Data presented above indicate that CAF mediated transcriptional suppression may involve intracellular signaling, leading to secondary gene expression. Reports by other groups, using CD8 culture supernatant, had indicated that the signaling molecule, STAT1, was involved in the process of suppression. We determined the role of STAT1 in the process of transcriptional suppression by CAF-containing exosomes from $CD8^+$ T cells. For this purpose, we utilized two fibrosarcoma cell, the STAT1-deficient U3A cell line and the constitutively STAT1-expressing U3AR cell line, both derived from the parental 2fTGH fibrosarcoma cell line. U3A and U3AR cells were incubated in medium only or medium supplemented with HIV-1-suppressing exosomes for 10 h. After incubation, all cells were liposome-transfected with an HIV-1 LTR-CAT gene reporter plasmid followed by mitogen induction of the LTR with PMA. After an 8-h PMA stimulation, intracellular CAT protein concentration was measured by ELISA. We observed no HIV-1 suppression

in STAT1-deficient U3A cells, but found 61% HIV-1 suppression in STAT1-expressing U3AR cells (Figure 12). This indicated that STAT1 was necessary for CAF-mediated suppression.

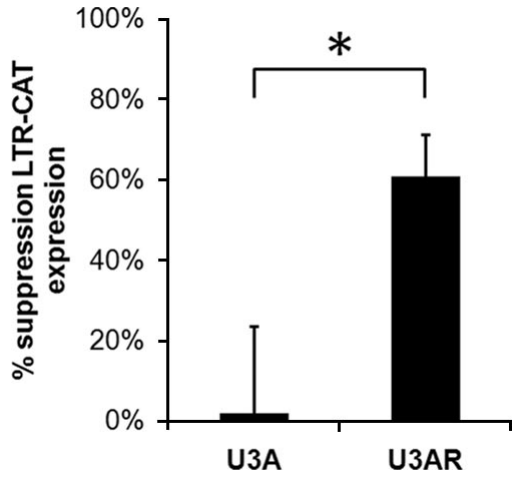


Figure 12: Requirement for STAT1 in CAF-mediated HIV LTR transcriptional suppression. Suppression of LTR-driven gene expression by CAF-containing exosomes is abolished in U3A (STAT1-deficient) cells, whereas it is restored in U3AR (STAT1 rescued) cells. Figure used with permission from the Journal of Virology [135]

6. Exosome fractions from culture supernatants of primary CD8 T cells can also suppress HIV- transcription: The HIV-1 suppression studies described above were conducted using Herpes virus saimiri (HVS)-transformed $CD8^+$ T cells. To confirm that the suppressive effects seen with the exosomes from transformed $CD8^+$ T cells were not an artifact of the transformation process, we cultured primary $CD8^+$ T cells from 2 therapy-naïve HIV-1 infected patients and isolated partially purified exosomes from the culture medium. Our results showed that both cell membrane as well as exosome fractions from

primary $CD8^+$ T cells could suppress HIV-1 replication in infected $CD4^+$ T cells, without affecting cell viability (Figure 13).

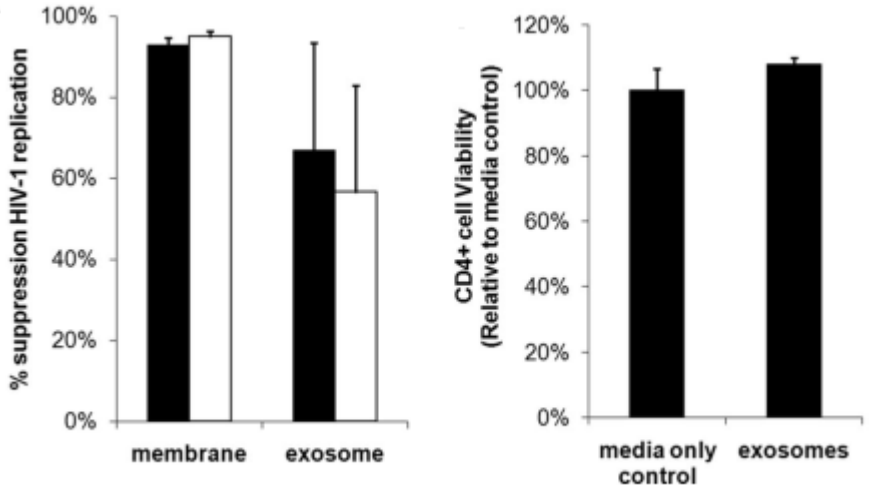


Figure 13: Membranes and exosomes from $CD8^+$ T cells of HIV-1 infected patients also suppress HIV-1 replication.

Cell membranes and exosome fractions from $CD8^+$ T cells of patient 1 (filled bars) and patient 2 (open bars), 2 therapy-naïve, HIV-1 infected subjects, can suppress HIV-1 replication in infected primary $CD4^+$ T cells (left panel), without affecting cell viability (right panel). Figure used with permission from the Journal of Virology [135]

4.4 CONCLUSIONS -1

Our overall aim was to determine the mechanism of action of CAF by which it suppresses HIV LTR-driven transcription. Since the identity of CAF is unknown, we used $CD8^+$ T cell- secreted exosomes as a source of CAF and to track the interactions of CAF-containing exosomes with target cells. Our results so far indicate that exosomes from $CD8^+$T cells can suppress HIV-1 LTR driven gene expression by inhibiting mRNA synthesis in $CD4^+$ target cells. We also observed that CAF-containing exosomes appear to interact with the TZM-bl target cells at the cell surface, as early as 10 minute after addition and did not seem to get internalized. However, maximal suppression was observed to occur about 12-16 hours after addition of CAF-containing exosomes to the cells. Interestingly, we found that exosomes can be removed within 5 minutes of addition to cells and still produce maximal suppression of transcription 12-16 hours later. These observations indicate that CAF in the exosomes probably triggers a signal cascade as soon as it encounters the cell and this signal cascade ultimately leads to transcriptional suppression of the LTR, 12 to 16 hours later. This was corroborated by findings that STAT1 appeared to be necessary for the process of suppression by CAF.

Hence, in the next section, we investigated the role of STAT1 in the transcriptional suppression of HIV-1 by CAF.

5.0 STAT1-INDEPENDENT SUPPRESSION OF HIV-1 TRANSCRIPTION BY PRIMARY CD8$^+$ T CELL-DERIVED ANTIVIRAL FACTOR

5.1 ABSTRACT

$CD8^+$ T cells can inhibit HIV-1 replication in infected $CD4^+$ T cells in a non-cytolytic manner by suppressing transcription from the viral promoter. The factor mediating this response, referred to as CD8 Antiviral Factor (CAF), and its mechanism of action are unknown. Previous reports, using HVS-transformed $CD8^+$ T cell lines, have shown the requirement for the transcription factor, STAT1, in CAF-mediated viral transcription suppression. Since STAT1 is not known to directly interact with the HIV-1 promoter, we attempted to determine upstream and downstream interacting proteins, to uncover novel pathways of viral transcription regulation by STAT1. We found Jak1 and Jak2 kinases to be involved in the STAT1 mediated suppressive process, in cells treated with CAF from transformed $CD8^+$ T cells ($CAF_{transformed}$). Jak1, Jak2 and STAT1 are components of the canonical interferon gamma (IFNγ) signaling pathway. Hence, we examined the role of IFNγ in STAT1-mediated suppression of HIV transcription. We found that CAF from primary CD8+ T cells from HIV-1 infected subjects ($CAF_{primary}$) contained 10-fold lower levels of IFNγ than $CAF_{transformed}$. Furthermore, IFNγ-depleted $CAF_{primary}$ retained the ability to suppress viral transcription in infected primary $CD4^+$ T cells and in STAT1-deficient cell lines. In contrast, IFNγ-depleted $CAF_{transformed}$ could not suppress viral transcription in the absence of STAT1. Our results indicate that CAF from primary $CD8^+$ T cells can suppress HIV-1 replication independent of STAT1 and that $CAF_{transformed}$ may not be a good substitute for $CAF_{primary}$

5.2 INTRODUCTION

$CD8^+$ T cells can control HIV-1 replication by non-cytolytic methods [21, 130]. The first non-cytolytic antiviral $CD8^+$ T cell response was described in Long Term Non-Progressors of HIV-1 infection [21]. The factor mediating this response was termed "CD8 Antiviral Factor" (CAF) [137]. Several features characterize the CAF-mediated response. First, there is no requirement for MHC restriction or contact between the $CD8^+$ T cell and the target cell [136, 176]. Second, CAF has been found to be effective against a wide range of HIV-1 clades, as well as HIV-2 and SIV and inversely correlates with the stage of disease [151, 153, 154, 156, 157, 160, 177, 178]. Third, CAF has been noted to suppress transcription from the viral promoter [135, 179-182]. Fourth, both R5 and X4 viruses can be equally well suppressed [155, 167]. Although many candidate non-cytolytic $CD8^+$ T cell factors have since been described [130-133], the identity and mechanism of action of CAF are as yet unknown.

The discovery of CAF was made by using primary $CD8^+$ T cells from human subjects. However, the short lifespan of primary T cells in culture, as well as the difficulties in obtaining a regular and consistent supply of fresh blood from infected patients prompted the use of Herpes virus saimiri (HVS)-transformed $CD8^+$ T cell lines for more detailed studies on CAF and its identity. Several of these studies have relied mainly on supernatants from transformed $CD8^+$ T cells, as a source of CAF[135, 162-166, 182-184]. A key result uncovered from these studies using cell lines knocked out for STAT1 was that STAT1 was involved in in the process of viral transcription suppression in target cells [165].

STAT1 is a well characterized signal transducer and regulates the expression of many genes involved in innate immunity, cell growth, differentiation and cell death [185, 186]. STAT1 can function as a signal transducer when it is phosphorylated, although it has recently been shown to have multiple signaling roles even in its unphosphorylated state [187]. Kinases known to phosphorylate STAT1 are the Janus kinases, Jak1, Jak2, Jak3 and Tyk2. Once phosphorylated, STAT1 has multiple interactions with many different proteins, including other STAT proteins, interferon regulatory factors (IRFs) [188], and other transcription factors such as SpI [189] , NFKB[190], BRCA I[191] and c-myc[192] (Figure 12). The genes induced by STAT1 in each case, depending on its interacting partners, may be different.

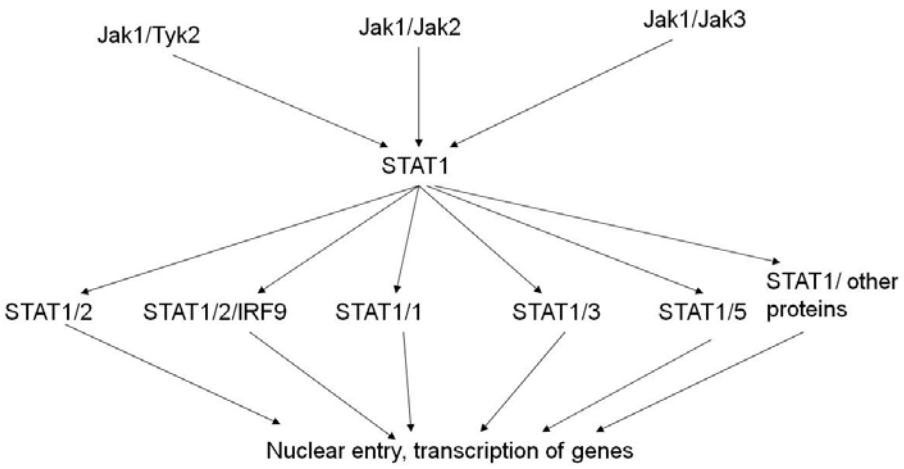


Figure 14: A schematic drawing showing the common upstream and downstream interacting partners of phosphorylated STAT1.

Among the most common inducers of STAT1 is interferon gamma (IFNγ) [193] . IFNγ is one of the key cytokines secreted by activated $CD8^+$ T cells and has multiple antiviral effects on cells. Recombinant IFNγ has been shown to be able to suppress HIV-1 transcription in HeLa based cell lines [194], although its effect on HIV-1 transcription in infected, primary $CD4^+$ T

cells is considered to be inconclusive, with reports of inhibition [195-197], enhancement [198] or no effect at all on viral transcription [199]. IFNγ interacts with its receptor IFNγR at the cell surface, triggering the recruitment of Jak2 to the receptor chain IFNγR2. Jak1, found to be constitutively associated with IFNγR1, and Jak2 are activated by trans-phosphorylation at key tyrosine residues. Phosphorylated tyrosines present attractive targets for the SH2 domain of STAT1, which is, in turn, recruited to and phosphorylated by Jak1 and Jak2 at its Y701 residue. Phosphorylated STAT1 dimerizes in response to IFNγ , enters the nucleus, becomes phosphorylated at the S727 site and is activated to function as a potent transcription factor, able to induce the expression of interferon-responsive genes [185].

Since STAT1 is not known to directly interact with the HIV-1 LTR promoter, we sought to determine the immediate upstream and downstream interacting partners of STAT1, in the hopes of uncovering novel antiviral pathways coincident with STAT1 activation. However, we found that the requirement for STAT1 in the suppressive process was exclusively found only when CAF from transformed $CD8^+$ T cell supernatant (henceforth referred to as $CAF_{transformed}$) was used. In contrast, CAF from primary $CD8^+$ T cells (henceforth referred to as $CAF_{primary}$) signaled in a STAT1-independent manner to suppress transcription from the HIV-1 promoter.

5.3 RESULTS

1. Role of STAT1 in the process of transcription suppression by CAF from transformed $CD8^+$ T cells:

Our previous studies indicated that STAT1 was necessary in the process of HIV-1 transcription suppression mediated by CAF from transformed $CD8^+$ T cell lines ($CAF_{transformed}$). Although STAT1 phosphorylation at the Y701 residue activates its function as a transcription factor [188], unphosphorylated STAT1 has also been shown to have important signaling roles [187, 200]. Hence, we determined the phosphorylation status of STAT1 in the presence of CAF by Western Blot. We found that STAT1 phosphorylation occurred upon $CAF_{transformed}$ treatment in TZM-bl cells (Figure 15)

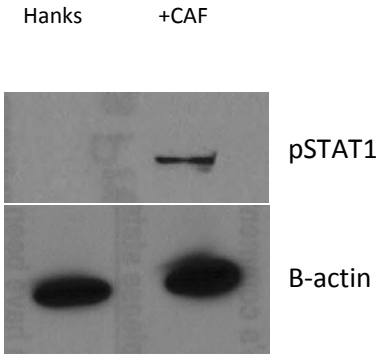


Figure 15: Phosphorylation of STAT1 in TZM-bl cells in response to $CAF_{transformed}$
TZM-bl cells were exposed to either Hanks or to $CAF_{transformed}$ for 15 minutes before being lysed and probed for phosphorylated STAT1

Next, we determined the time-and dose-response kinetics of STAT1 phosphorylation by quantifying the level of phosphorylated STAT1 using the Luminex assay. We found that there was a time and dose dependence to STAT1 phosphorylation by $CAF_{transformed}$ in TZM-bl cells (Figure 16A, 16B. Maximal phosphorylation of STAT1 was seen about 30 minutes after addition of $CAF_{transformed}$. We also assessed the dynamics of STAT1 phosphorylation by $CAF_{transformed}$ in primary $CD4^+$ T lymphocytes. We treated primary $CD4^+$ T cells either with varying doses of $CAF_{transformed}$ for a fixed period of time or with a fixed dose of $CAF_{transformed}$ for varying periods of time. We found that STAT1 phosphorylation in primary cells was also time- and $CAF_{transformed}$ dose- dependent (Figure 16C, 16D). The percentage of phosphorylation in CD8- cells increased from ~0.7% to ~5% when the dose of $CAF_{tranformed}$ was increased from 0.5% vol/vol to 15% vol/vol.

2. Role of upstream and downstream interacting partners of STAT1:

Since STAT1 was phosphorylated in response to $CAF_{transformed}$, we next sought to determine the role of upstream and downstream interacting partners of STAT1 in CAF-mediated suppression. Since the most common kinases that phosphorylate STAT1 are the Jak kinases, Jak1, Jak2, Jak3 and Tyk2 [201] and STAT2, STAT3, STAT5 and IRF9 are known STAT1 interacting partners (Figure 12), we tested the importance of Jak1, Jak2, STAT2, STAT3, STAT5 and IRF9 in CAF-mediated transcription suppression by using gene knock-out cell lines and by probing the phosphorylation status of these proteins in response to $CAF_{transformed}$. The knock-out cell lines that we used were U1A (Tyk2 knock out), U2A (IRF9 knock out), U4A

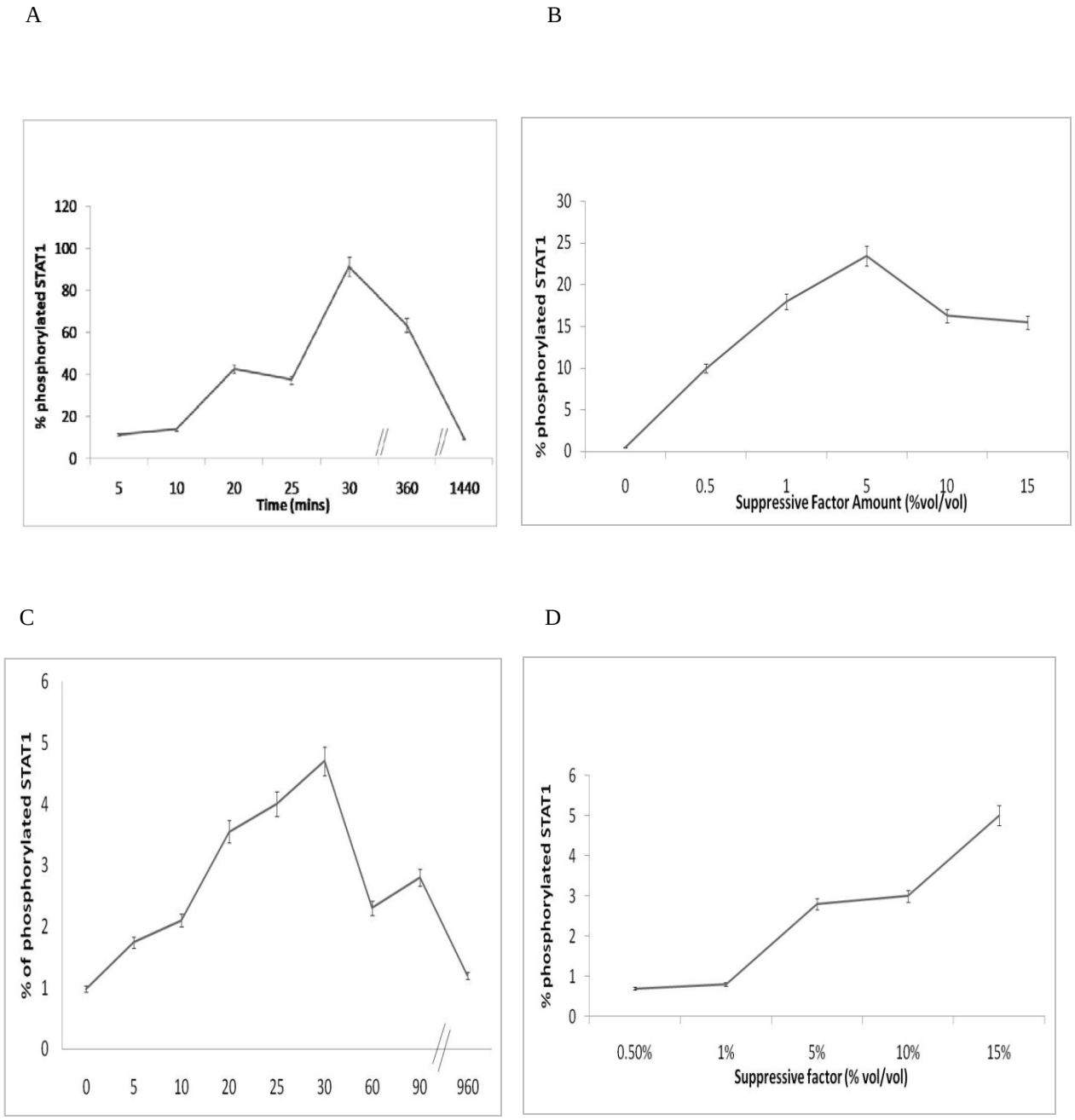


Figure 16: Time- and dose- dependence of phosphorylation of STAT1 in response to $CAF_{transformed}$

The time and dose dependence of phosphorylation of STAT1 in TZM-bl cells (A and B) upon exposure to $CAF_{transformed}$ and in primary $CD4^{+}$T cells (C and D) were determined by Luminex bead assay. The y-axis is the percentage of phosphorylated STAT1 (pSTAT1) in the cells.

(Jak1 knock out), U6A (STAT2 knock out) and G2A (Jak2 knock out), previously described [202-204]. In experiments involving these knock-out cell lines, we pre-treated each of the cell types with $CAF_{transformed}$, and then transiently transfected the cells with a LTR-luciferase reporter plasmid. After PMA stimulation, we measured luciferase expression from LTR in the CAF-treated cells, and compared it to the control-media treated cells. We found that the cells knocked out for Jak1 and Jak2 proteins only suppress LTR driven reporter gene expression to ~7-8%, indicating that these proteins were necessary for suppression. However, ~95% suppression of luciferase expression from the LTR-luc plasmid upon $CAF_{transformed}$ treatment still occurred in the cell lines U1A, U2A and U6A, deficient in Tyk2, IRF9 and STAT2 respectively, indicating that these proteins were not involved in the suppression process (Figure 17).

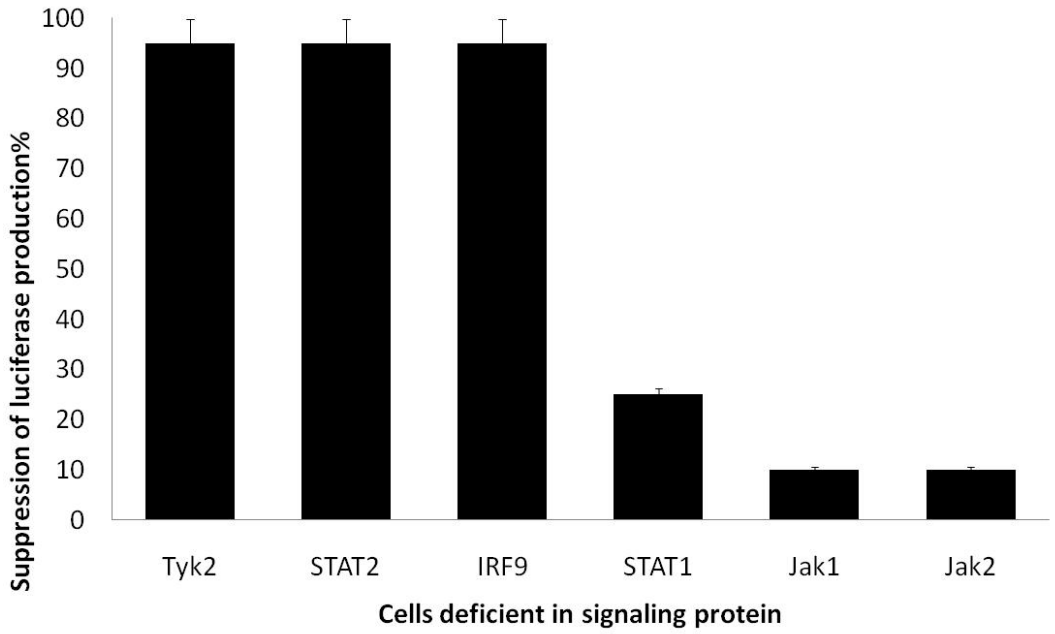


Figure 17: Suppression of LTR-gene expression by $CAF_{transformed}$ in cells deficient for different proteins

Cells deficient in each of the signaling proteins above were treated with either $CAF_{transformed}$ or Hanks control and then transfected with LTR-luc plasmid. Luciferase production from the LTR was measured after PMA stimulation of the cells. The degree of suppression of LTR gene expression in CAF treated cells was calculated relative to the control cells

Western blots of TZM-bl cell lysates showed that STAT3 and STAT5 were not phosphorylated after treatment of these cells with CAF, whereas in control TZM-bl cells treated with IL2, phosphorylated STAT3 and STAT5 was readily detected (Figure 18). Since Jak3 phosphorylation also results in the concomitant phosphorylation of STAT3 [201] and STAT3 phosphorylation was not detected upon CAF treatment, we concluded that Jak3 was not involved in CAF-mediated transcription suppression. Thus, the main signaling partners of STAT1 found to be involved in the suppression of gene expression from the HIV-1 LTR, were Jak1 and Jak2.

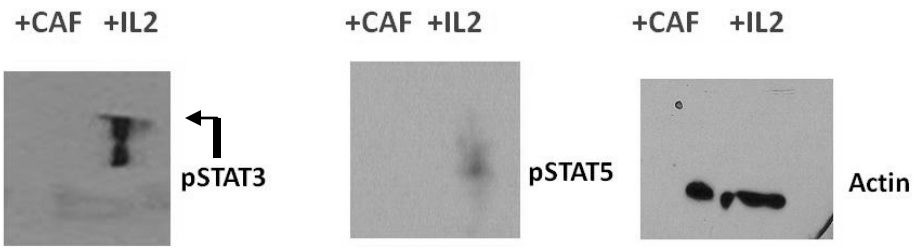


Figure 18: STAT3 and STAT5 are not phosphorylated in response to $CAF_{transformed}$.

The phosphorylation states of STAT3 and STAT5 were studied by Western blots in TZM-bl cells treated with $CAF_{transformed}$ or a positive control (IL2) for 15 minutes

3. Role of IFNγ in the CAF-mediated signaling process:

Thus far, we have determined, using $CAF_{transformed}$, that the signaling proteins necessary for the suppressive action by CAF were Jak1, Jak2 and STAT1. These signaling proteins are also important constituents of the canonical interferon gamma (IFNγ) signaling pathway. High IFNγ levels in $CAF_{transformed}$ would explain the requirement for Jak1, Jak2 and STAT1 in the signaling process.

Therefore, we next measured the level of IFNγ in supernatants from the transformed $CD8^+$T cells as well as from primary $CD8^+$T cells from HIV-1 infected individuals. We found that samples from transformed $CD8^+$T cells indeed had consistently higher levels of IFNγ (>4000pg/mL) than supernatants derived from most of primary $CD8^+$T cells (<200pg/mL) (Table 5-1).

Table 5-1: IFNγ levels in CD8 culture supernatants.

Source of CAF	IFNγ levels (pg/mL)
Patient 1	12
Patient 2	16
Patient 3	74
Patient 4	61
Patient 5	186
Patient 6	794
TG 1	3050
TG 2	7290
TG 3	8050
TG 4	10880

IFNγ in $CAF_{primary}$ from 6 different HIV-1 infected patients and in $CAF_{transformed}$ from 4 different batches of the transformed cell line TG was measured by ELISA

Next, we sought to determine if IFNγ depletion from the $CD8^+$T cell culture supernatants would affect the ability of $CAF_{transformed}$ to suppress LTR- gene expression. IFNγ was depleted from culture supernatants by serial incubation on IFNγ antibody- coated plates. The percentage of depletion by this method was nearly always greater than 90% (see Table 5-2 for IFNγ levels

before and after depletion in transformed CD8 and primary CD8 culture supernatant samples).

Table 5-2: IFNγ levels in culture supernatants of $CD8^+$ T cells before and after depletion

S no	Sample	IFNg before depletion (pg/mL)	IFNg after depletion (pg/mL)	% depletion
1	TG sample 1	1262	99.7	92
2	TG sample 2	696	26	96
3	TG sample 3	305	3	99
4	HIV Pt 1	397	34	91
5	HIV Pt 2	98	1.7	98
6	HIV Pt 3	93.2	9.5	90
7	HIV Pt 4	29	0	100
8	HIV Pt 5	15	1.8	88
9	HIV Pt 6	8	4	50
10	HIV Pt 7	5	0	100

IFNγ was measured in the supernatants of transformed $CD8^+$ T cells, TG, from 4 different batches and primary $CD8^+$ T cells from 7 different patients

Following IFNγ depletion, we compared the ability of the depleted samples to suppress LTR mediated reporter gene expression with those which had undergone a control-depletion with nonspecific IgG antibody. We found that IFNγ-depleted $CAF_{transformed}$ could no longer suppress LTR-transcription in TZM-bl cells, as compared to the non-depleted CAF samples (Figure 19).

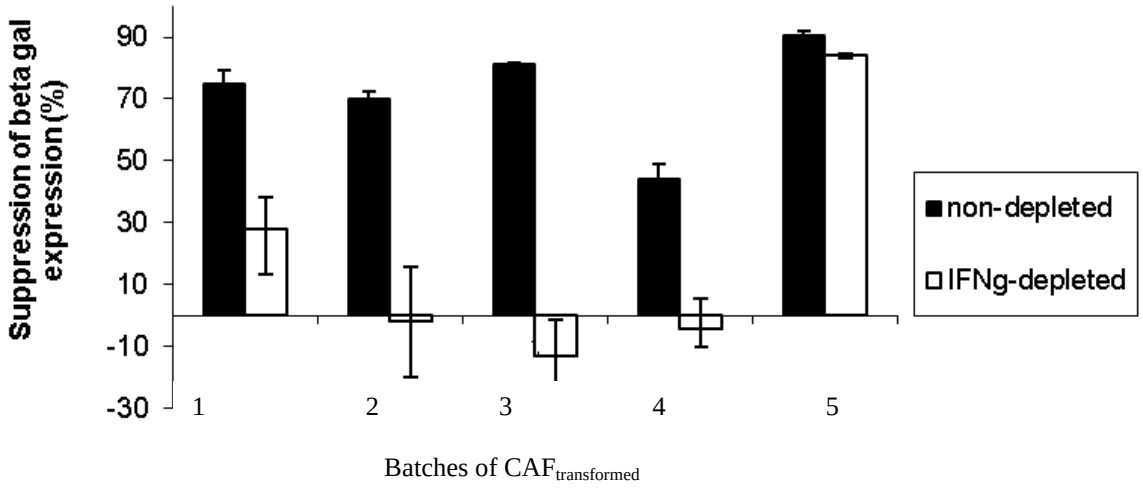


Figure 19: Depletion of IFNγ abolishes suppressive ability of $CAF_{transformed}$ in TZM-bl cells.

5 different batches of $CAF_{transformed}$ were IFNγ-depleted and their ability to suppress LTR transcription was compared to non-depleted controls in TZM-bl cells

To determine if suppression of HIV-1 replication in primary, infected $CD4^+$ T cells by $CAF_{transformed\ CD8}$ was affected by IFNγ depletion, we cultured the infected $CD4^+$ target cells in the presence of either IFNγ-depleted $CAF_{transformed}$ or non-depleted, control $CAF_{transformed.}$ In contrast to the results seen with TZM-bl cells, we found that IFNγ depleted $CAF_{transformed}$ could suppress p24 production to nearly the same extent as the non-depleted samples in acutely infected primary $CD4^+$ T cells (Figure 20).

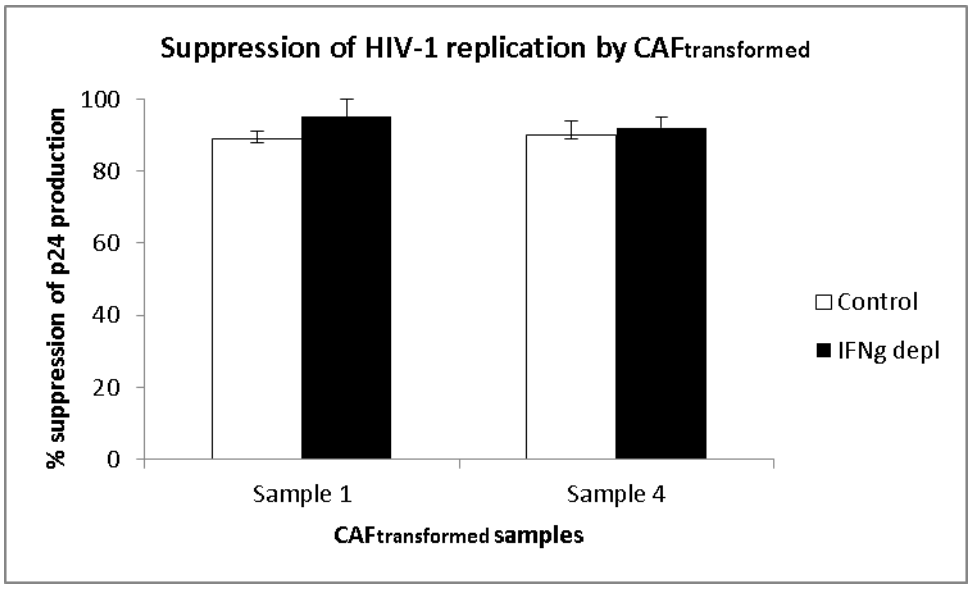


Figure 20: Depletion of IFNγ does not affect ability of $CAF_{transformed}$ to suppress HIV-1 replication in primary $CD4^+$ T cells.

IFNγ -depleted ("IFNg-depl") and non-depleted ("control") $CAF_{transformed}$ CD8 were assayed for their ability to suppress HIV-1 transcription acutely infected primary CD4+ T cells

Next, we sought to determine the effect of IFNγ depletion on the suppressive ability of $CAF_{primary}$. IFNγ-depleted $CAF_{primary}$ showed no change in the ability to suppress HIV-1 p24 production, compared to the non-depleted controls, in acutely infected primary $CD4^+$ T cells, in 8 out of 9 cases (Figure 21). The suppression of p24 production by IFNγ depleted $CAF_{primary}$ was also dose dependent (Figure 22).

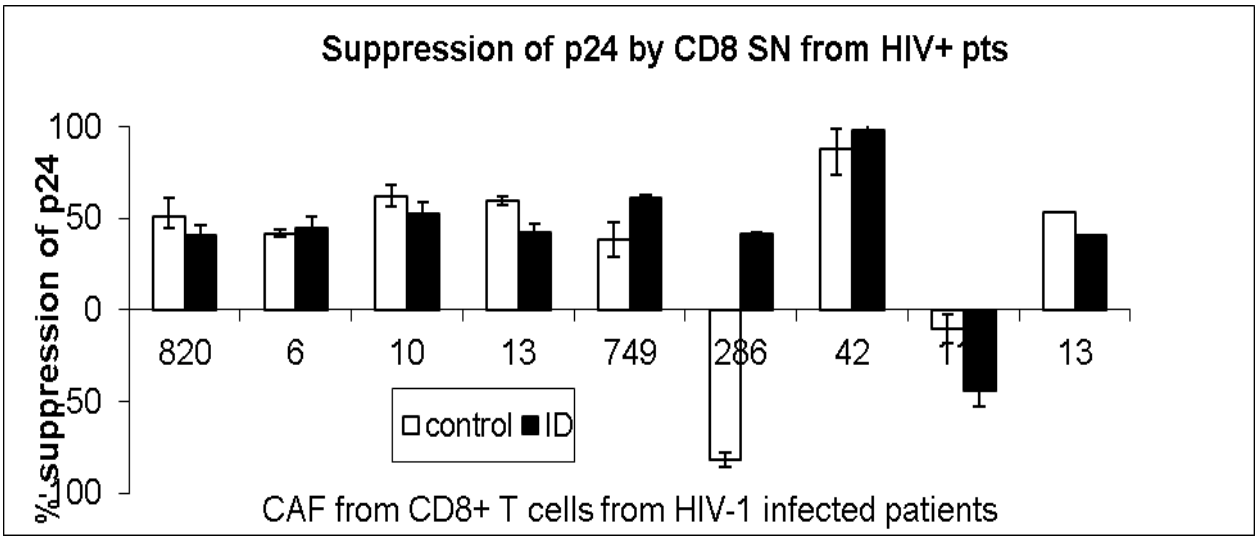


Figure 21: Depletion of IFNγ from $CAF_{primary}$ does not affect its ability to suppress HIV-1 replication in primary $CD4^+$ T cells.

Non-depleted ("control") and IFNγ-depleted ("ID") samples from 9 different HIV-1 infected patients were assayed for their ability to suppress HIV-1 replication in primary $CD4^+$T cells, by measuring p24 levels

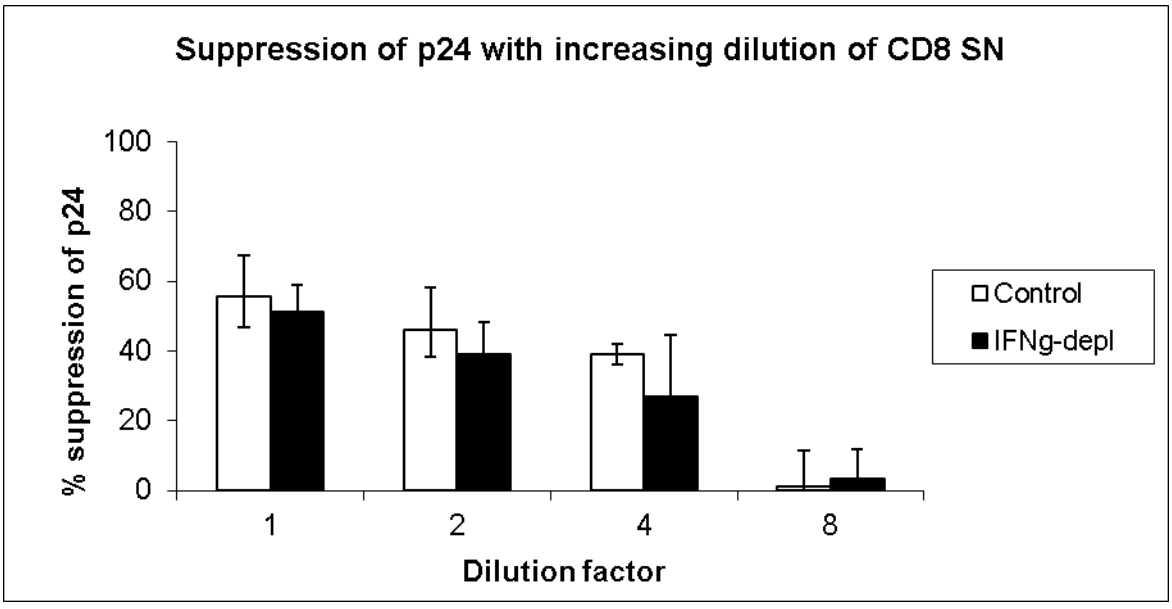


Figure 22: Dose dependence of suppression of HIV-1 replication by $CAF_{primary}$.

Dilutions of non-depleted ("control") or IFNγ-depleted ("ID") supernatants from one of the patient $CD8^+$ T cells were used to examine the dose dependence of the suppression of HIV-1 replication in infected $CD4^+$ T cells.

These results indicate firstly that, transformed $CD8^+$T cell supernatants do contain significantly higher levels of IFNγ than primary $CD8^+$T cell supernatants. These results also indicate IFNγ depletion does not affect the ability of either $CAF_{transformed\ CD8}$ or $CAF_{primary\ CD8}$ to suppress HIV-1 replication in infected $CD4^+$ T cells. We also found that TZM-bl cells may not be sensitive to factors other than IFNγ that mediate LTR suppression, thus implying that the choice of assay for measuring suppressive ability of CAF is important.

4. Role of STAT1 in suppression by IFNγ-depleted CAF from primary $CD8^+$ T cells:

Our results so far have shown that culture supernatants of transformed $CD8^+$ T cell contain significantly higher levels of IFNγ than that of primary $CD8^+$T cells. Since $CAF_{transformed}$ signals via Jak1, Jak2 and STAT1, which are constituents of the IFNγ signaling pathway, we investigated whether STAT1-mediated viral suppression was due to IFNγ present in this source of CAF.

To address this, we once again used the STAT1-deficient and rescued cell lines, U3A and U3AR, which had been used previously to establish the importance of STAT1 in viral transcription suppression. U3A and U3AR cells were treated with non-depleted or IFNγ-depleted $CAF_{primary}$ and $CAF_{transformed}$, following which, the cells were transiently transfected with LTR-luc plasmid. After PMA stimulation, luciferase expression from the LTR was measured. Although suppression by $CAF_{transformed}$ still required STAT1 (Figure 23), we observed no difference between the suppression of gene expression from the LTR in U3A and U3AR cells in all samples of IFNγ-depleted $CAF_{primary}$, indicating that STAT1 had no role to play in the process of viral transcription suppression when $CAF_{primary}$ was used (Figure 24).

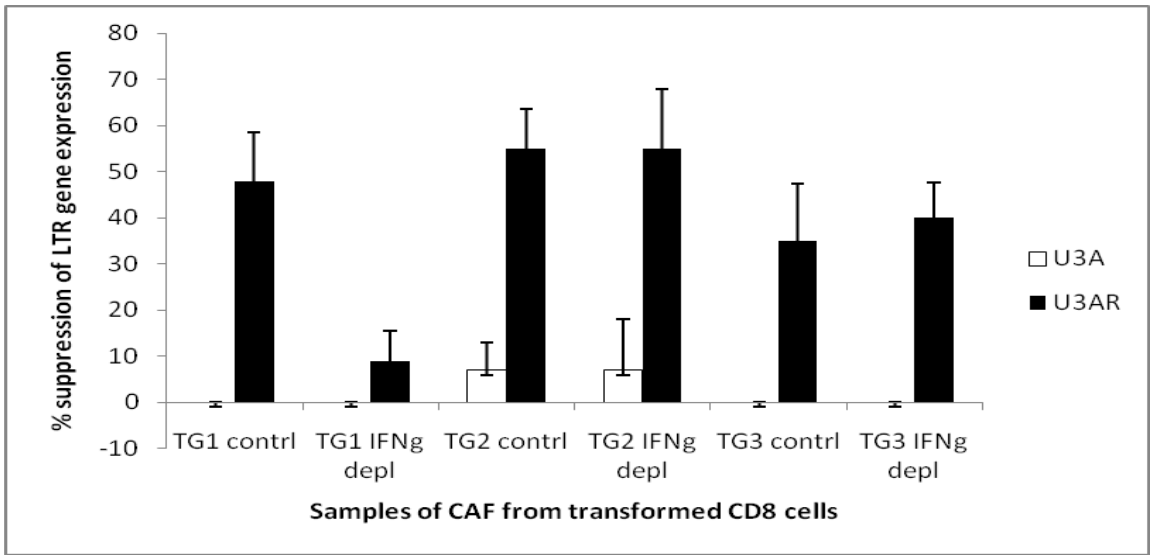


Figure 23: Requirement for STAT1 for suppression by non-depleted and IFNγ-depleted $CAF_{transformed}$

STAT1 deficient (U3A) and rescued (U3AR) cell lines were used to analyze the role of STAT1 in suppression of LTR driven transcription mediated by 3 batches (TG1-TG3) of non-depleted ("contrl") or IFNγ-depleted ("IFNg depl") $CAF_{transformed}$

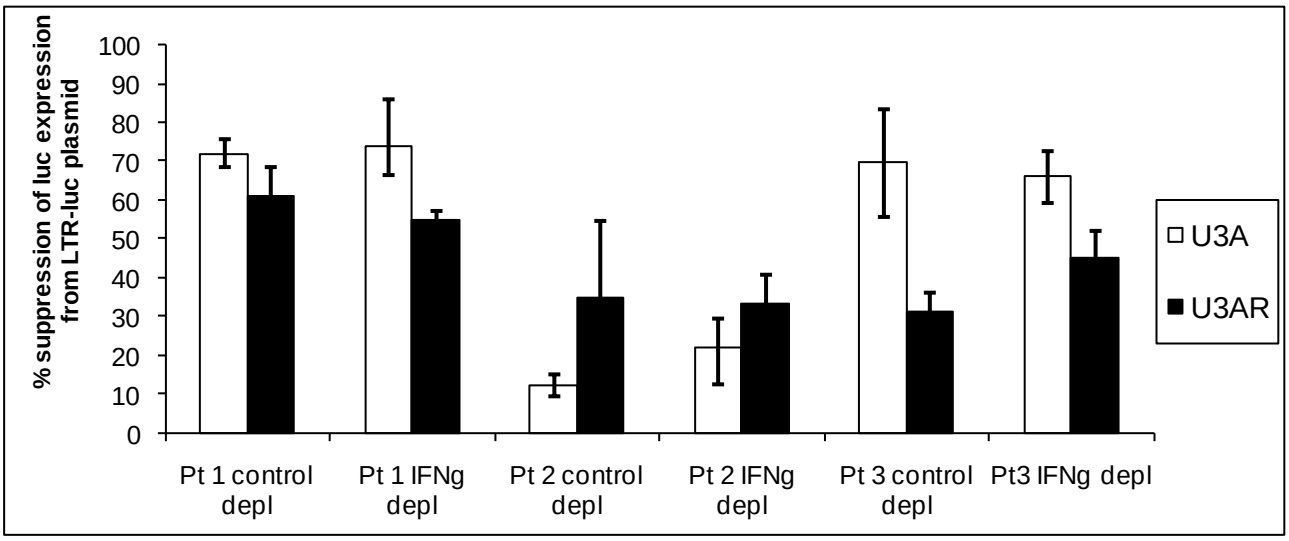


Figure 24: STAT1 independent suppression by non-depleted or IFNγ-depleted $CAF_{primary}$

STAT1 deficient (U3A) and rescued (U3AR) cell lines were used to analyze the role of STAT1 in CAF-mediated suppression of LTR driven transcription. Both cell types were exposed to non depleted ("control depl") or IFNγ-depleted ("IFNg depl") $CAF_{primary}$ from 3 different HIV-1 infected patients (Pt1- Pt 3) and then transfected with LTR-l

MTT assays indicated no loss in cell viability after incubation with supernatant samples (data not shown). Overall, our results indicate STAT1-independent suppression by $CAF_{primary}$.

5. Role of PI3K, MAPK and other common signaling proteins in $CAF_{primary}$-mediated suppression:

Previous investigations have shown that significant levels of suppression of viral transcription are only seen about 12-16 hours after addition of CAF to $CD4^+$ cells (Figure 7 and [135]). Our data also indicates that CAF might trigger a intracellular signal soon after addition to cells, which ultimately leads to viral transcription suppression 12-16 hours later. This indicates the necessity for expression of intermediate proteins which might directly suppress transcription from the LTR. Since STAT1 was not involved in the process of suppression by $CAF_{primary}$, we investigated whether any other protein could be considered a suitable marker for CAF activity. This included proteins like the kinases p38 MAPK, JNK or PI3K which are components and key regulators of multiple signaling pathways in the cell. It might be possible that CAF signaling could result in the phosphorylation of some of these proteins. Hence, we examined the phosphorylation status of 10 different proteins in acutely HIV-1 infected, primary $CD4^+$ T cells that had been treated for various intervals of time with $CAF_{primary}$. These proteins were p38 Mitogen Activated Protein Kinase (p38 MAPK), Phosphotidyl inositol 3 kinase (PI3K), Phospholipase C γ2 (PLCγ2), Jun N terminal kinase (JNK), Janus kinase 3 (Jak3), Signal Transducer and Activator of Transcription 3 (STAT3), Interleukin 15 receptor alpha chain (IL15Ra), Fibroblast Growth Factor Substrate (FRS2) and the adaptor proteins Crk, and Tec kinase. Some of these proteins are known to modulate HIV-1 transcription. For example, PI3K and IL15 are known to inhibit HIV-1 transcription and enhance CAF activity, respectively [205,

206]. JNK, p38 MAPK and FRS2 are known to activate HIV-1 transcription [207, 208]. Other proteins, like STAT3, are known to be intermediates in pathways that generate HIV-1 transcription inhibitors [209]. PLC-γ2 and Tec kinase are involved in regulating the levels of intracellular calcium, which has modulating effects on HIV-1 transcription [210-212]. The adaptor protein, Crk, is an intermediate in many MAPK pathways and connects multiple stimuli to the JNK pathway [213].

Since this entire set of proteins is activated by phosphorylation, we determined the phosphorylation status of these proteins at various time after CAF treatment. For this purpose, acutely HIV-1 infected, primary $CD4^+$ T cells were exposed to IFNγ depleted or control CAF from primary $CD8^+$T cells of 3 HIV-1 infected subjects for 10 minutes, 20 minutes, 12 hours or 42 hours and probed for tyrosine phosphorylation. Concomitantly, we also measured the suppression of p24 production in acutely infected $CD4^+$ T cells by the same CAF samples and confirmed that the degree of suppression was about 60-70% in all cases. We saw no consistent significant difference in phosphorylation of any of the 10 proteins studied at any of the time points, compared to PBS-only controls (Figures 25A-D). Thus, we were unable to find a molecular marker for the suppressive activity of $CAF_{primary}$ with the methodology used.

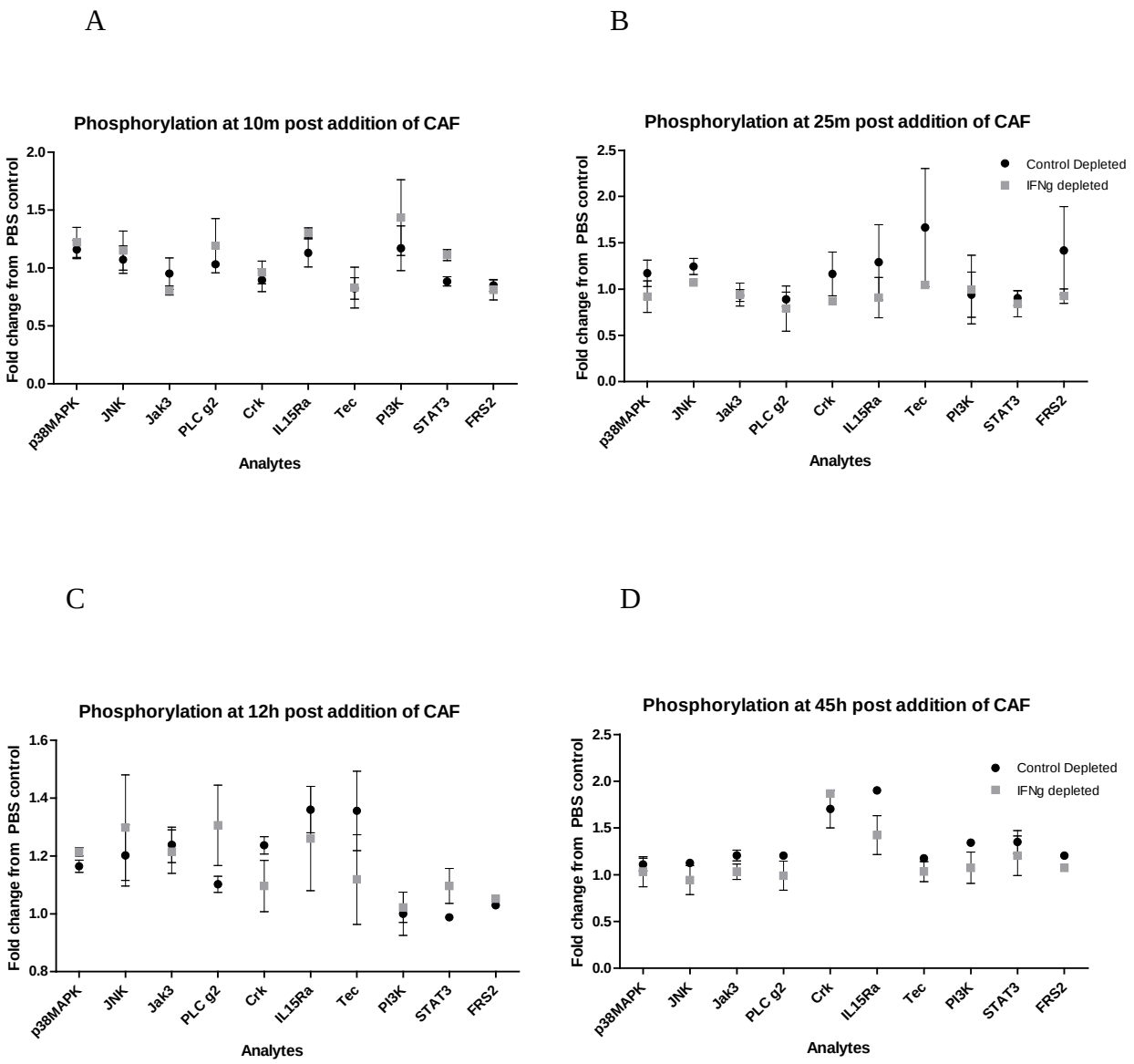


Figure 25: Role of PI3K, p38MAPK and other common signaling proteins in suppression mediated by $CAF_{primary}$.

Exogenously infected primary $CD4^+$T cells were exposed to either non-depleted or IFNγ-depleted $CAF_{primary}$ (referred to as "control" or "ID" in figure) from 3 different HIV-1 infected patients or media control for 15m, 25m or 12h and then lysed and processed for measuring phosphorylation, using Epiquant technology (Millipore, Billerica, MA). The phosphorylation of 10 different proteins was measured against a standard cocktail of phosphorylated peptides provided by the manufacturer at 10 min (A), 25 min (B), 12 hours (C) or 42 hours (D) of CAF treatment.

5.4 CONCLUSIONS – 2

Our results from Specific Aim 1 indicated the involvement of an intracellular signaling cascade triggered by CAF, which might ultimately lead to viral transcriptional suppression. Previous work, by us and by others, had indicated the importance of STAT1 in this process. Hence, we examined the role of STAT1 in the process of CAF-mediated viral transcription suppression.

We found that Jak1 and Jak2 were the upstream interacting partners of STAT1 in the CAF-mediated suppressive process. Since Jak1, Jak2, and STAT1 are components of the IFNγ signaling pathway, we examined the role of IFNγ in suppression. Our results indicate that transformed $CD8^+$ T cell culture supernatants contain IFNγ at levels about 10 times higher than that from primary CD8 cells. The high levels of IFNγ lead to misleading results about the role of Jak1, Jak2, and STAT1 in the process of transcription suppression by $CAF_{transformed}$. However, even in the absence of IFNγ, $CAF_{transformed}$ shows a requirement for STAT1, that is not indicated by $CAF_{primary}$, indicating that differing IFNγ levels may not be the only reason for the opposite results with $CAF_{transformed}$ and $CAF_{primary}$. Figure 26 summarizes the main differences between the two sources of CAF. Since $CAF_{transformed}$ and $CAF_{primary}$ also exhibited different suppressive abilities in TZM-bl cells and primary CD4+ T cells, we summarized the major differences. Table 5-3 summarizes the ability of various sources of CAF to suppress in the two different assay sytems, the TZM-bl transcription and CD4+ T cell HIV-1 acute infection assays.

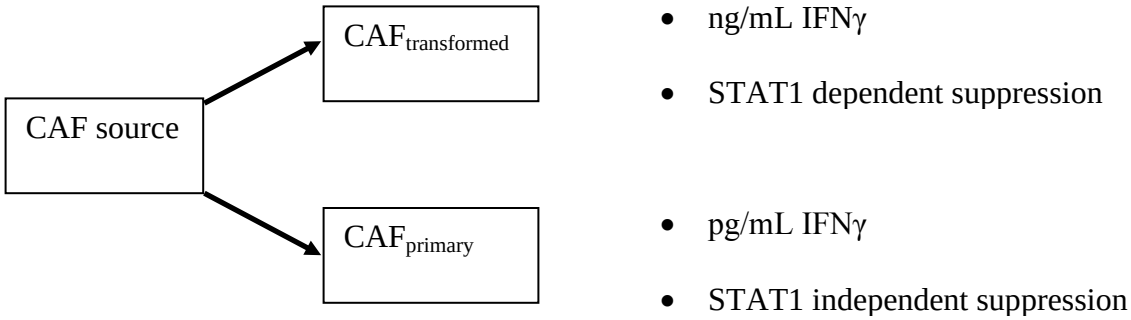


Figure 26: Summary of differences between $CAF_{transformed}$ and $CAF_{primary}$

Table 5-3: Summary of suppressive ability of CAF sources in different assays

Assay/CAF source	**TZM-bl transcription assay** (% suppression)	**$CD4^+$ T cell acute infection assay** (% suppression)
$CAF_{transformed}$	>90% [135]	>90% [135]
IFNγ-depleted $CAF_{transformed}$	~ 0 (Figure 19)	~90% (Figure 20)
$CAF_{primary}$	Not performed	Variable (Figure 21, 22)
IFNγ-depleted $CAF_{primary}$	Not performed	Same as non-depleted (Figure 21,22)

Since phosphorylation is one of the most common ways by which signals are transduced within cells, we then sought to determine other signaling molecules that might be phosphorylated in response to $CAF_{primary}$. We determined the phosphorylation of 10 different proteins, which were known to either directly regulate HIV-1 transcription or were components of pathways that converged on the LTR. However, none of these proteins were seen to be consistently phosphorylated in response to $CAF_{primary}$. Our results indicate the requirement for a STAT-1 independent signaling pathway in the process of HIV-1 transcriptional suppression by CAF from primary $CD8^+$ T cells.

We next turned our attention to the HIV-1 promoter, the Long Terminal Repeat (LTR), in the hopes of uncovering a region that was necessary for the suppressive process, and which might provide additional clues as to the signaling molecules involved.

6.0 IDENTIFICATION OF THE REGION ON THE HIV-1 LONG TERMINAL REPEAT RESPONSIBLE FOR TRANSCRIPTIONAL SUPPRESSION BY THE CD8 ANTIVIRAL FACTOR

6.1 ABSTRACT

The CD8 Antiviral Factor (CAF) is known to suppress transcription from the HIV Long Terminal Repeat (LTR) promoter, in a non-cytolytic manner. However, the region on the LTR that is crucial for the suppressive activity of CAF is unknown. Serial deletions of the HIV-1 LTR indicated a short ~90 bp segment containing the 3 SpI sites and TATA box on the LTR is sufficient for CAF-mediated transcriptional suppression. Furthermore, we found that the 3SpI-TATA segment, with no other upstream modulators or enhancers, could independently support transcription and suppression. Thus, the changes in protein binding occurring on the HIV-1 minimal promoter, spanning the 3 SpI sites and TATA box of the LTR, likely lead to transcriptional suppression by CAF.

6.2 INTRODUCTION

The aim of this part of the project is to identify the region on the HIV-1 promoter, the Long Terminal Repeat, which was crucial for the process of transcriptional suppression by CAF.

The studies described in earlier sections of this thesis imply that CAF may mediate its suppressive effects through the production of an intermediate protein which might lead in alteration of transcription factors binding to the viral promoter, the HIV-1 Long Terminal Repeat (LTR), leading to decreased transcription. The HIV-1 LTR consists of 3 regions: U3, R and U5. The core promoter of HIV consists of 3 non-identical GC-rich SpI binding sites (-78 to -46) along with the TATA box (-28 to -20), on the U3 region [214, 215]. Immediately upstream of the SpI binding sites are 2 sites for the transcription factor NFKB (-105 to -79), an important enhancer and modulator of HIV transcription [216]. Other upstream enhancer elements include NFAT, ApI, COUP-TF, Ets and USF [217-219]. Downstream of the TATA box (+3 to +59) is the HIV Tat protein binding region, known as the Transactivating Region (TAR) [215, 220](Figure 27). Transcription begins with the binding of cellular transcription factors NFKB, SpI, TATA binding protein (TBP) and RNA Pol II to the cognate sites, upon which nascent mRNA is produced. A characteristic hairpin loop with bulge structure is formed by the nascent mRNA, which is known as the TAR loop. The viral protein Tat binds to TAR, recruiting the transcription elongation factor pTefb [221, 222]. The cdk9 component of pTefb is a cyclin dependent kinase. It phosphorylates the C terminus of RNA Pol II, increasing its processivity and resulting in the elongation of the viral mRNA [222]. Since most of the transcription regulatory sites are located in the U3 and R regions, we concentrated on these regions to determine the target site for transcriptional suppression by CAF.

A recent paper by Bonneau et al [223]examined the effect of deletions and inactivating point mutations in individual transcription binding factor sites on the LTR on CAF activity. Specifically, the authors performed mutational analyses to investigate the role of the transcription factors NFAT (-256 to -234) and ApI (at -351 to -327 and -310 to -289), the Interferon Stimulatory Response Element (ISRE, which is a binding site, situated at +198 to +219, for interferon regulatory factors, IRFs), and IL2-homology Purine Rich Response Element (PRRE, at -274 to -256). They also studied the role of the 2 NFKB sites and the 3 SpI sites by deleting them separately and disrupted the TAR-Tat axis by mutational inactivation of the Tat gene and TAR. Their results showed that full length HIV-1 molecular clones bearing these individual mutations and deletions could still exhibit suppression of replication in the presence of CAF. Hence, we considered the possibility that more than one transcription factor binding site on the LTR might be involved in the process of the CAF-mediated HIV transcriptional suppression. Therefore, instead of introducing point mutations as described by Bonneau et al, we serially deleted LTR constructs, to identify the minimal region responsible for CAF-mediated transcriptional suppression. Our data suggest that a 90bp region containing the 3 SpI sites and the HIV TATA box is sufficient for transcriptional suppression by CAF.

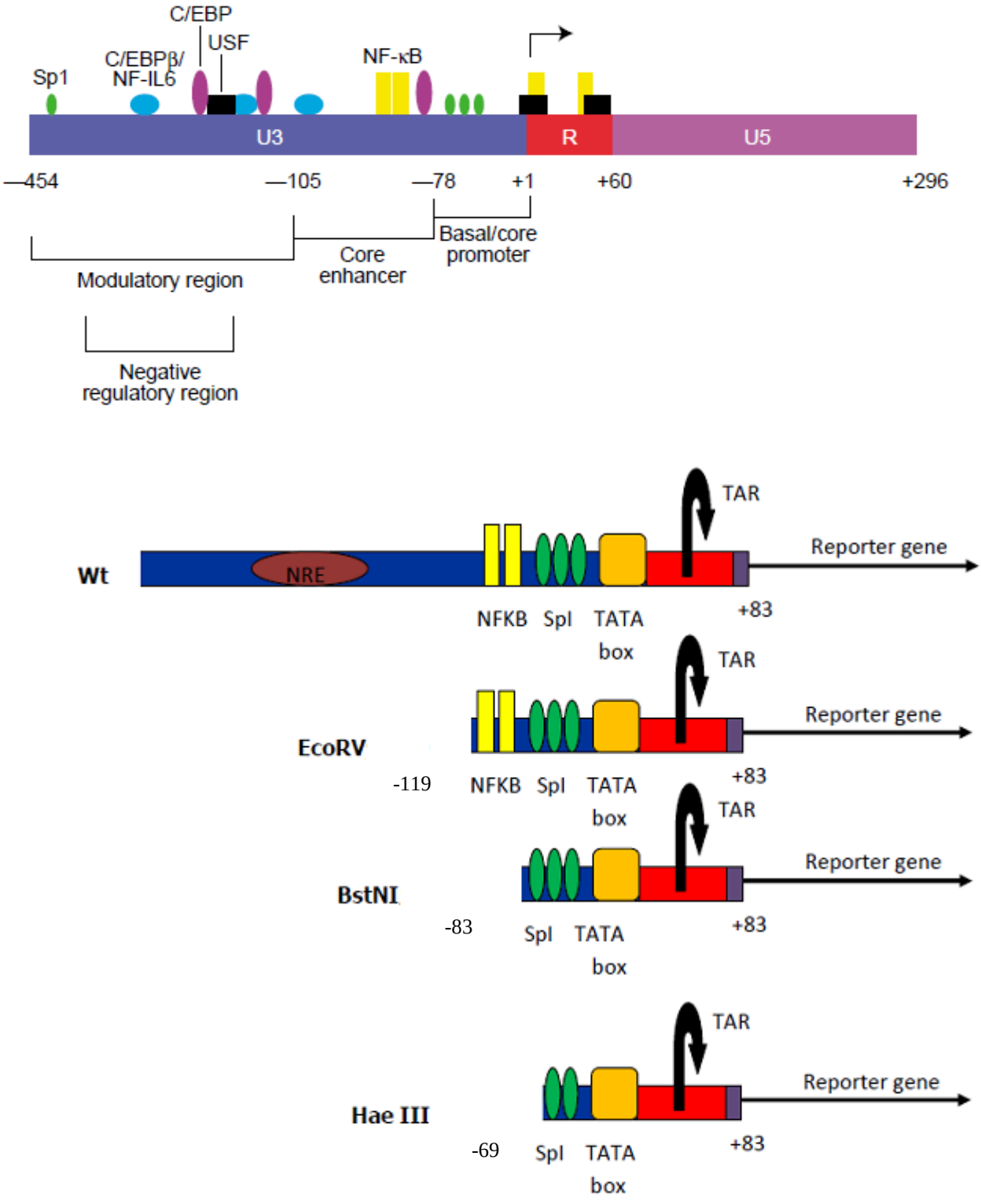


Figure 27: Schematic of the HIV-1 LTR and LTR deletion constructs.
Upper panel shows cartoon of the entire 650bp HIV-1 LTR and is used with permission from Expert Reviews in Molecular Medicine

6.3 RESULTS

To explore the possibility that more than one TF binding site may be acting together to suppress LTR driven transcription, we focused on the U3 and R regions in our study. The main segments of the U3 region were the Negative Regulatory Element (NRE) (-340 to -184), the 2 NFKB sites, the 3SpI sites, and the TATA box (Figure 27).

1. Measurement of transcriptional suppression in serially deleted constructs of the LTR: To study if there are multiple TF binding site acting in conjunction with each other to initiate CAF-mediated suppression of transcription from the LTR in the presence of CAF, we used LTR constructs which had been serially deleted of key TF binding sites. These constructs were the full length Wt-LTR, the EcoRV construct (containing deletion of the NRE), the BstNI construct (containing deletions up to the SpI sites), and the Hae III construct (deletion up to the SpI (II) site) as shown in Figure 24. Each of these constructs was transfected into 293T cells, pretreated with either CAF or media control, and then stimulated with PMA. LTR driven reporter gene expression was then measured. We found that CAF was able to suppress reporter gene expression from all of these constructs (Figure 28)

   These results indicate that the region encompassing the -69 to +83 of the LTR is sufficient for the transcription suppressive action of CAF.

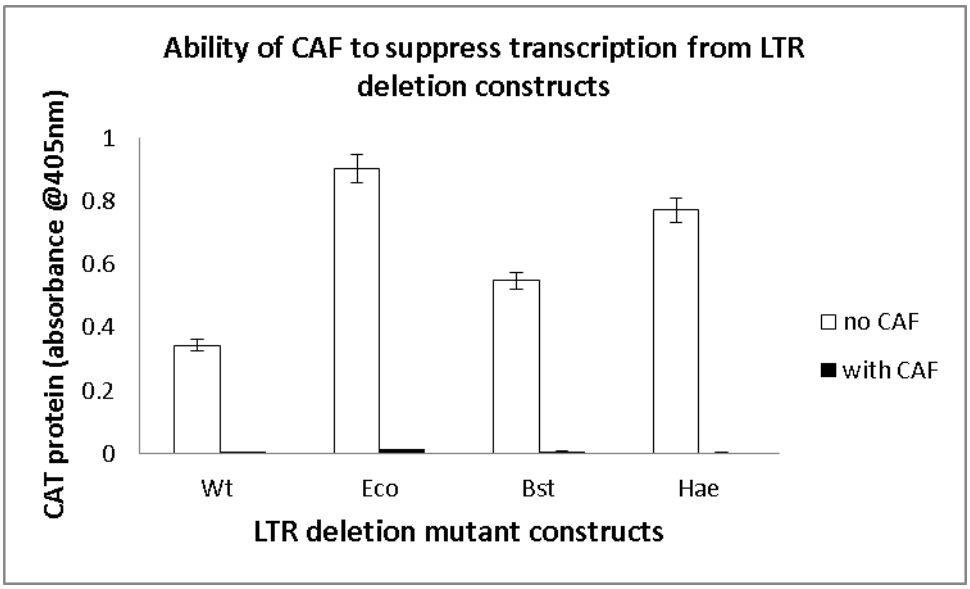


Figure 28: Suppression of transcription by CAF from various LTR deletion constructs

CAT protein production from various LTR deletion constructs in the presence and absence of CAF. Wt refers to the full length (-454 to + 83) LTR upstream of the CAT reporter gene. The positions of the LTR truncations by each of the restriction enzymes, EcoRV, BstNI, and HaeIII are as shown in Figure 27.

2. Role of the TAR loop in conferring susceptibility to CAF: The results above indicate that the smallest segment of the LTR that was able to do was the HaeIII segment, containing the 1st 2 SpI sites, the TATA box and TAR region. However, we decide focused our attention on all three of the SpI sites, because the SpI sites of the LTR have been shown to be crucial for modulating transcription and SpI (III) is especially important because it can recruit activating and repressing transcription factors [224-226]. Hence, we focused our attention on the BstNI construct which contains the 3 SpI sites, HIV TATA box and TAR regions.

The TAR region is a very attractive candidate target for CAF action. It has a unique bulge-loop structure and is well-conserved across all clades of HIV-1 [227]. If TAR were the target for CAF, it would help explain the specificity of CAF for HIV. Previous investigations on the region of the LTR necessary for CAF action, created

inactivating, point mutations of the TAR to disrupt the Tat-TAR axis [223]. But if the structure of TAR were somehow important for CAF action, point mutation alone might not indicate the importance of TAR in suppression. Critical interactions between the TAR, Tat and pTefb take place on the bulge-loop structure on TAR [228, 229] Hence, we deleted the bulge-loop region of TAR (shaded in Figure 29A, right panel) from the BstNI segment, to form a new construct called BstΔTAR, to evaluate any changes in susceptibility to CAF.

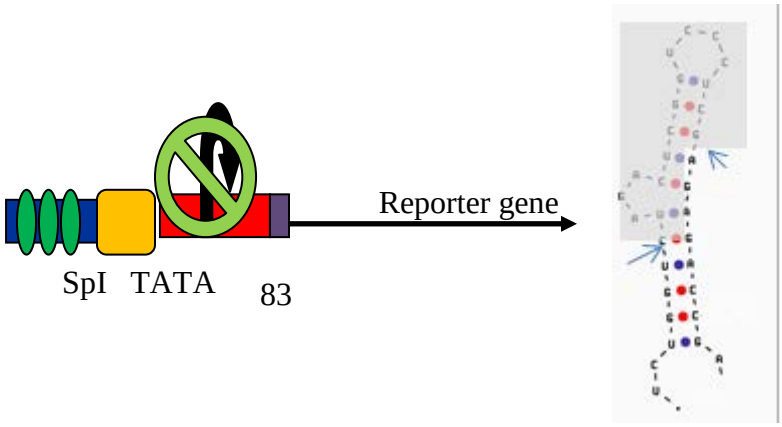


Figure 29: Deletion of the TAR region in the LTR construct.

Schematic of TAR deletion (left panel). The secondary structure of wt TAR (right panel). The arrows point to the two sites in the TAR where the deletion was made. The shaded region shows the region that was deleted

Contrary to expectations, the TAR deleted construct was still transcriptionally active and was suppressed by CAF transcription from the LTR (Figure 30). We reasoned that the absence of any upstream LTR elements in the BstΔTAR construct may be a reason why TAR deletion still supported transcriptional ability of the construct. We concluded that the TAR element is not the target region for transcriptional suppression by CAF.

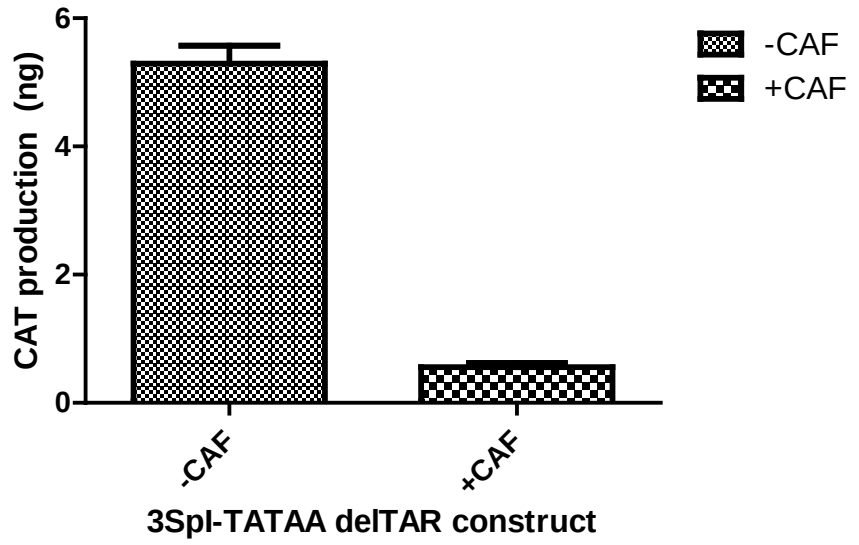


Figure 30: Suppression of reporter gene expression from the BstΔTAR construct in the presence of CAF

BstdelTAR construct was transfected into 293T cells that had been pre-treated with CAF. CAT reporter production was measured after stimulation of the LTR with CAF.

3. Role of the SpI sites in CAF-mediated suppression: We next sought to determine if any of the SpI sites might be important in the suppressive process. For this purpose, we first deleted each SpI site individually or in pairs, in the presence of the deleted TAR, to check for changes in response to CAF. The schematic in Figure 28 details the deletions made.

As shown in Figure 31, deletion of the SpI sites had different effects on LTR driven transcription. Deletion of SpI (III) alone, in the absence of TAR, made the construct transcriptionally inactive, highlighting the importance of this site as well as TAR in HIV-1 transcription, as has been reported earlier [230]. Deletion of SpI(I) or SpI (II) alone did not affect the ability of the construct to transcribe upon activation LTR-driven gene expression or get suppressed when exposed to CAF. However, deletions of both SpI (I) and SpI (II) made the construct transcriptionally inactive.

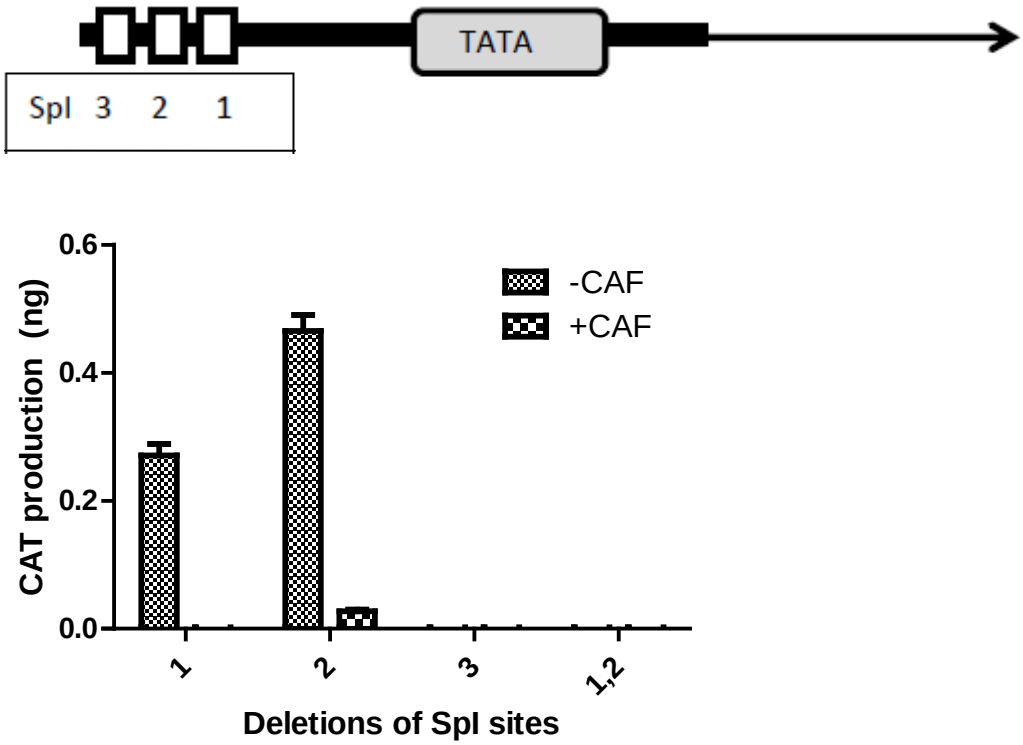


Figure 31: Suppression by CAF of transcription from SpI-deletion constructs of the HIV-1 LTR. Deletions of the SpI sites, individually or pairwise, on the 3SpI-TATA segment of the HIV-1 LTR were made. Deletions affected transcription from this segment, although suppression by CAF was seen as long as transcription was supported

To confirm that the observed results were a product of SpI site inactivation and not because the of the deletion process itself, we next inactivated the SpI sites by point

mutation. Inactivation of the SpI sites, either individually or in pairs, was done by replacing key G residues in the SpI binding sites with T. Mutation of the SpI sites did not transcriptionally inactivate the construct, in accordance with previous reports [231]. However, mutation of the SpI (III) site did reduce the ability of the construct to drive transcription, to ~500 RLU compared to the ~1500 RLU of luciferase production by the single mutations in the remaining SpI sites. While inactivation of SpI (I) or SpI (II) alone did not appear to significantly reduce transcription, mutation of both SpI (I) and SpI (II) simultaneously reduced transcription by nearly 50%. Mutation of SpI (I) or SpI (II) with SpI (III) reduced the transcription ability even further (Figure 32). All constructs were able to support transcription, albeit to different degrees, compared to the deletion mutants (Figure 30). However, the results with the SpI point mutations broadly corroborate with those obtained using the SpI deletion mutants

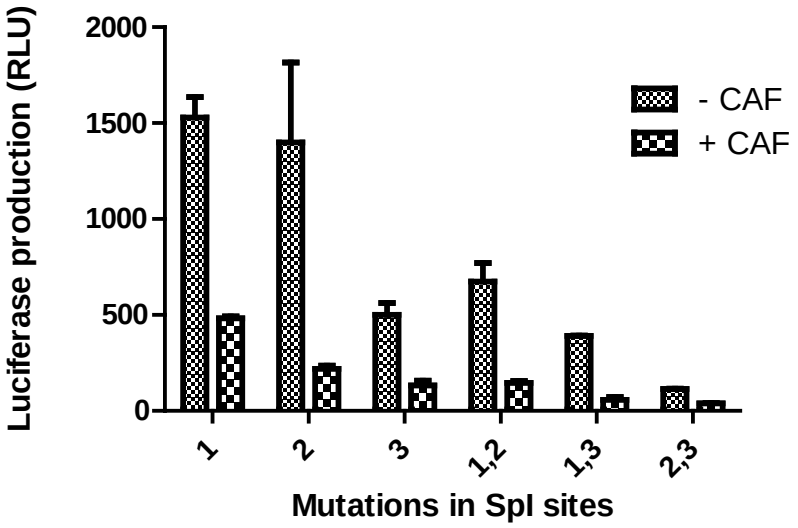


Figure 32: Suppression by CAF of transcription from SpI-point mutation constructs of the HIV-1 LTR.

Inactivating point mutations of the SpI sites, individually or pairwise, on the 3SpI-TATA segment of the HIV-1 LTR were made. Inactivation by mutation affected transcription from this segment, although suppression by CAF was seen as long as transcription was supported

While these constructs gave further insight into the role of the SpI sites in HIV transcription, we still could not parse out which sites were crucial for CAF suppressive activity. This was especially so, because the processes of transcription and suppression of transcription were inextricably linked. The generation of an "ideal" transcriptionally active construct, resistant to CAF action, could not be achieved by inactivating the SpI sites alone or in pairs.

All the data described above indicate that the 3SpI-TATA box is the smallest segment of the HIV LTR that can independently transcribe and get suppressed by CAF. We noticed that the Wt, EcoRV, BstNI and HaeIII LTR constructs carrying the 3SpI-TATA box and TAR (used in Figure 27 and described in [168, 232]) contained upstream GAL4 and adenovirus E1B sequences. We first sought to investigate if removal of these upstream GAL4 sequences would have any effect on the ability of the constructs to suppress HIV transcription when exposed to CAF. Secondly, we wanted to determine if the 3SpI-TATA segment of HIV LTR could independently transcribe and get suppressed in response to CAF, in the absence of any other enhancer or promoter elements and TAR sequence. Hence, we inserted the HIV minimal promoter construct upstream of the luciferase reporter gene in a promoter-less vector, pGL4.10 and determined if it was transcription competent. We found that that the 3SpI-TATA segment could be inserted into a promoterless reporter gene construct and it could still undergo LTR-driven transcription. Addition of CAF from 2 different batches of transformed $CD8^+$ T cell culture supernatants as well as CAF from primary $CD8^+$ T cells from an HIV infected individual suppressed transcription from this construct efficiently (Figure 33). Since results from

Specific Aim 2 have indicated that interferon gamma (IFNγ) may also play a role in suppressing transcription from the HIV LTR, we determined if IFNγ-depleted supernatant from $CAF_{transformed}$ and $CAF_{primary}$ samples could also suppress LTR driven transcription. Our results show that IFNγ depleted CAF could also suppress transcription from the 3SpI-TATA construct, and indicate, once again, that there is indeed a non-IFNγ component in $CD8^+$ T cell culture supernatants that can suppress LTR transcription (Figure 33). Further deletions of the SpI inactivated the construct (data not shown), corroborating our previous results with the SpI deletion constructs, shown in Figure 31

.

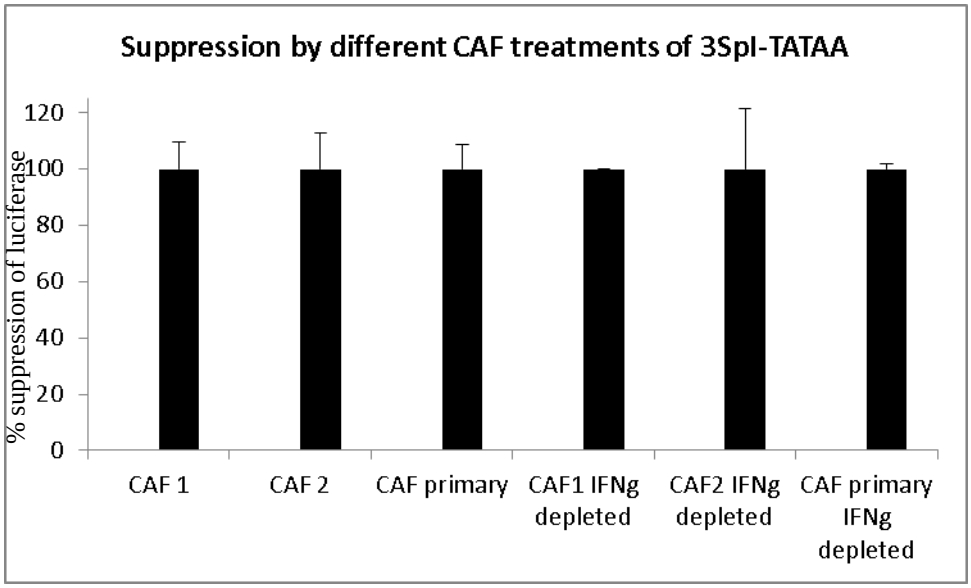


Figure 33: Suppression of transcription from the 3SpI-TATAA LTR segment by different CAF treatments

The 3SpI-TATA segment is able to transcribe independently and suppress transcription in the presence of 3 different sources of CAF, either used "as is" or IFNγ depleted: CAF1 and CAF2 are from 2 different batches of supernatant from transformed CD8, CAF primary is from primary CD8 cells from an HIV-1 infected patient.

6.4 CONCLUSIONS- 3

We sought to determine the region on the HIV-1 LTR that was crucial for the process of viral transcriptional suppression by CAF. Serial deletions and point mutation analyses of the LTR indicated that this region was ~90 bp in length and contained 3 SpI sites and the TATA box. The 3 SpI sites and TATA box of the LTR constitute the minimal promoter of HIV-1. Our results also indicate that each individual SpI site contributes differently to the process of transcription, depending upon the presence or absence of the TAR loop. In the presence of TAR, the third SpI site, SpI (3) can be deleted without any effect on the ability of the construct to support transcription. In the absence of TAR, however, the deletion of SpI (3) abolishes the ability of the construct to support transcription. Thus, the only region that could independently support transcription as well as respond to the suppressive effects of CAF was the 3SpI-TATA of the HIV-1 LTR.

7.0 OVERALL DISCUSSION

CAF is a non-cytolytic CD8 cell secreted factor that can suppress HIV transcription. The identity and mechanism of action of CAF are unknown. The overall aim of this project was to determine the mechanism of action of CAF. Towards this goal, we formed three specific aims: a) to examine the interaction between CAF and its target cells, b) to identify the signal cascades triggered by CAF which would lead to transcriptional suppression from the HIV-1 promoter, the LTR and c) to identify the region of the HIV-1 LTR necessary for CAF action.

HIV-1 suppression was observed by CAF present both in a soluble form in the culture supernatant as well as in a membrane-bound form on the surface of intact CD8 T cells. Since the identity of CAF is unknown, it was not possible to directly observe its interaction with its target cells. However, previous work from our lab showed that CAF was present on small membrane vesicles, known as exosomes, which were secreted by $CD8^+$ T cells. Hence, we used these exosomes as markers for CAF and evaluated their interactions with target $CD4^+$ cells. Since CAF was associated with the exosomes, it was likely that we could extrapolate the interactions of CAF with the target cells from those of the exosomes with the cells.

We observed that CAF-containing exosomes interacted with target cells at the cell surface and that this interaction took place as early as 10 minutes after addition of exosomes to

cells. Our studies using labeled CAF-containing exosomes showed, by confocal microscopy, that exosomes were not internalized into the cells and their interactions, even after 12 hours of exposure to cells, are restricted to the cell surface. Our studies with the kinetics of CAF activity indicated that cells required a time interval of 12-16 hours between CAF addition and suppression of gene expression. These results suggested the involvement of some intracellular signaling cascade in the process of suppression and indicated a role for the signal transducer and transcription factor, STAT1 (as shown in Figure 12).

A few caveats are present in this study using the CD8 T cell-derived exosomes to study CAF interactions. Since we did not study the interaction of "control" exosomes which lack CAF, it is possible that the surface-restricted interactions observed between the CAF-exosomes and target CD4+ cells are solely because of the property of the exosomes and not specific to CAF. Furthermore, as the size of exosomes is as little as 100nm, it is likely that the exosomes in our confocal microscopy data represented by the red dots actually are clumps of exosomes, instead of individual vesicles. The process of fluorescently labeling exosomes with Cy5 dye might cause them to clump or adversely affect their shape in other ways. Whether the process of labeling the exosomes impacts the interaction of exosomes with target cells is unknown. However, given the current lacunae in our knowledge of CAF and its identity, we have no other means to examine the interactions of CAF.

The second specific aim of this project investigated the role of STAT1 in the process of transcription suppression. Since its identity is unknown, the study of CAF has mainly relied on unpurified sources of CAF. While we used CD8 exosomes as a CAF source and as a tool to track

its interactions in specific aim 1, isolating exosomes is a labor-intensive process. Previous results from our lab had also shown that different CAF sources, such as CD8 exosomes or CD8 culture supernatant, suppressed HIV-1 transcription in very similar ways. Hence, we used culture supernatants from transformed $CD8^+$ T cells for the remainder of this project.

Culture supernatants from transformed $CD8^+$ T cells have been used in studies on CAF for about 2 decades [162, 164]. They have provided an ideal alternative to the problems faced with using primary $CD8^+$ T cells, namely, short life span and difficulty in obtaining large quantities of blood samples from HIV-1 infected patients. Previous studies, using supernatants from transformed $CD8^+$ T cells, have shown that the signal transduction protein, STAT1, is necessary for the process of viral transcription suppression. However, the process of transformation has been known to lead to pleiotropic changes in the cell. The secretion of cytokines, such as IL4, IL17 and IL26, has been shown to be altered by the transformation process [233-235]. In fact, IL17 and IL26 were discovered in HVS-transformed $CD8^+$ T cells. Changes have also been noted in the activation of protein kinases, such as Lck, a protein crucial in T cell development and activation, as also in the expression of certain miRNAs, after transformation [236, 237]. While there has been no study to examine the secretion of interferon gamma (IFNγ) from these cells, it is not surprising that transformed cells produce more of this cytokine. The process of transformation of $CD8^+$ T cells by HVS can activate them. The HVS protein, Tip 484, has been shown to activate p56 lck [233, 237], a kinase known to interact with and activate STAT1 and STAT3 proteins [237]. STAT1 and STAT3 have been found to be crucial for the activation of the transcription factors T-bet and Bcl3, both of which induce the production of IFNγ [238, 239]. Thus, it is not surprising that we found higher concentrations of

IFNγ in supernatants from the transformed, as compared to primary $CD8^+$ T cells from HIV-1 infected patients.

IFNγ, by itself, has been known to suppress HIV-1 transcription both in HeLa-derived cell lines and in acutely infected $CD4^+$ T cells [195-197]. One way by which this could occur is through the action of c-myc. IFNγ can regulate the expression of c-myc through STAT1 phosphorylation [192]. C-myc and SpI are known to cooperatively bind HDAC1 to repress HIV-1 transcription [240]. Thus IFNγ, present in cell culture medium, could very well contribute to the process of HIV-1 transcription suppression. However, using IFNγ depleted CAF, we have shown that both $CAF_{transformed}$ and $CAF_{primary}$ still retained their ability to suppress HIV-1 transcription/replication (Figure 12C and 12 D). Therefore, IFNγ is not the sole contributor of the antiviral activity present in $CD8^+$ T cells. In our experiments using STAT1 deficient cell lines and IFNγ-depleted CAF, we observed that HIV-1 suppression by $CAF_{primary}$ did not require STAT1 (Fig 13B), but $CAF_{transformed}$ still required STAT1 to suppress HIV-1 even after IFNγ depletion. This observation corroborated our earlier finding that there was little correlation between pSTAT1 levels and the degree of HIV-1 suppression in cells treated with $CAF_{primary}$ (Fig17 A and B).

Our results contradict those reported by Chang et *al*. In their article, Chang et *al*. showed evidence for the necessity of STAT1 for the process of HIV-1 transcription suppression, using $CAF_{transformed}$ [165]. They also found the IFNγ-inducible protein, IRF1, to be activated in response to CAF. The investigators had considered the possibility of a confounding role played by IFNγ. Finding a wide range of IFNγ concentrations in the three HVS-transformed $CD8^+$ T cell

lines that they tested, they immune-depleted IFNγ from their CAF preparations by using neutralizing antibody to the cytokine and concluded that IFNγ is not a confounder in interpreting the results on CAF. We have found that a single incubation with neutralizing antibodies was ineffective in removing all the IFNγ in a sample, especially if the starting concentration of IFNγ in the sample was high (unpublished observations).The residual amount of IFNγ in the sample after a single neutralization step might be still high enough to display considerable antiviral effects. Thus, incomplete removal or inactivation of IFNγ from the culture supernatants might explain the requirement for STAT1 and the activation of IRF1 observed by Chang et al [165]. Using a serial incubation strategy on IFNγ antibody-coated ELISA plates, to deplete IFNγ consistently by more than 90% (Table 4-2), we were able to show that IFNγ-depleted $CAF_{primary}$ did not require STAT1 for its suppressive ability (Figure 13 B). The residual IFNγ in these samples was too low to affect suppression (data not shown).

However, our results also show that IFNγ may not be the sole reason for the differences in STAT1 requirement seen between $CAF_{transformed}$ and $CAF_{primary}$. We found that IFNγ-depleted $CAF_{transformed}$ samples still require STAT1 for efficient suppression of gene expression from a transfected LTR-luciferase reporter (Figure 13 A). So while IFNγ might certainly be a confounding presence in $CAF_{transformed}$, leading to misinterpretations about the role of STAT1 phosphorylation and the requirement for Jak1 and Jak2, and might explain the activation of IRF1 observed by Chang et *al*., it is only one of possibly many factors that differ between $CAF_{transformed}$ and $CAF_{primary}$.

An interesting result, which might provide another reason for the differences seen between this paper and the findings by Chang et *al*., is the response of TZM-bl cells to IFNγ and IFNγ- depleted supernatants. IFNγ is known to suppress HIV LTR driven transcription in HeLa based cell lines [194, 241]. Our studies indicate that TZM-bl cells are sensitive enough to IFNγ that removal of IFNγ from $CAF_{transformed}$ abolished suppression of viral transcription. Evidence for differences in the response of TZM-bl cells and PBMCs was recently presented in a study that showed LPS, which exhibits potent anti-HIV activity in PBMCs by inducing the expression of beta chemokines, has no effect on TZM-bl cells. Yet culture supernatant from LPS stimulated PBMCs exhibited potent suppression of HIV transcription in TZM-bl cells due to the effect of PBMC-secreted IFNγ, rather than any beta chemokines [241]. This study, like ours, suggests the higher sensitivity of TZM-bl to IFNγ than to other inhibitory factors.

A caveat in our results is that we used the culture supernatant from only one HVS-transformed $CD8^+$ T cell line in our investigations. It is possible that other HVS-transformed $CD8^+$ T cell lines secrete less IFNγ into the supernatant. Indeed, Chang et *al* did find variations in the levels of IFNγ between different transformed $CD8^+$ T cell lines. However, our results from the primary $CD8^+$ T cells from 9 HIV-1 infected patients clearly demonstrate a non-IFN γ component for CAF, which signals in a STAT1 independent manner.

There have been many soluble factors described since the discovery of CAF, which display noncytolytic suppressive activities [130, 132, 242-244]. Some of these factors, like prothymosin alpha or HIV Resistance Factor (HRF) identify with CAF in that they too suppress viral transcription [132, 243]. But neither of these proteins display the other key characteristics

associated with CAF: prothymosin alpha acts on infected macrophages, and not $CD4^+$ T cells, while HRF is a factor secreted by $CD4^+$ T cells, and not $CD8^+$ T cells. CAF is thought to be secreted in small quantities into the extracellular medium by $CD8^+$ T cells. This has made the investigations of CAF, particularly the elucidation of its molecular identity, difficult. Transformed $CD8^+$ T cells allow for the production of CAF-containing supernatants in quantities not attainable by primary $CD8^+$ T cell cultures. However, our results suggest that the differences between transformed and primary $CD8^+$ T cell culture supernatant are large enough to result in misinterpretations.

Proteomic analysis using increasingly versatile mass spectrometery techniques may help in both identification of the components of CAF, as well as unravel the different mechanisms of viral transcription suppression. In applying such methods, an expanded phosphoproteome analysis might aid identification of novel pathway markers for CAF-induced signaling. Hence, we examined the phosphorylation status of 10 different proteins, known to be either directly involved in the transcriptional regulation of HIV-1 (such as PI3K, PLCg2, JNK, p38 MAPK and IL15), or were components of pathways that could regulate viral transcription (such as FRS-2, Crk, Tec kinase, Jak3 and STAT3). We found no consistent increase in phosphorylation of any of these proteins at 4 different time points after addition of 3 different sources of $CAF_{primary}$ to infected $CD4^+$ T cells. It indicates that either none of these 10 proteins are involved in suppression, or that we were not able to capture a phosphorylation event of some of these proteins using our methodology.

Our studies also suggest that CAF might well be multifactorial: different cytokines and chemokines might act in concert to suppress viral transcription and replication. Perhaps there is an additive, or even a synergistic, effect of the smaller suppressive abilities of individual cytokines, like IFNγ, or chemokines. Investigations of the region of the viral promoter necessary for transcription suppression and the proteins that bind to it in the presence and absence of CAF may also yield clues to the signaling pathways that suppress viral transcription.

In the last specific aim, we determined the region of the promoter necessary for transcriptional suppression of HIV-1 by CAF. While the precise mechanism by which CAF inhibits HIV-1 transcription from the LTR is currently unknown, there are various ways by which the viral RNA produced from the integrated proviral promoter could be reduced upon the addition of CAF to the infected cell. These may include degradation of viral mRNA in the cytoplasm by RNA interference (RNAi) mechanisms, changes in the mRNA export from the nucleus or changes in transcription factor (TF) binding at the promoter. Other studies have shown that the first possibility, that of mRNA degradation in the cytoplasm by Dicer and Drosha-mediated RNA interference, is not likely in the mechanism of CAF action, since siRNA treatment against Dicer and Drosha does not inhibit CAF activity [245]. While there are many viral proteins, such as influenza NS1 protein, which could act by inhibiting RNA export from the nucleus, there has been no evidence to indicate this to be a key method of CAF action [246]. Hence, we focused our attention on the third possibility, namely, alterations in the binding of transcription factors to the viral promoter leading to inhibition of transcription. We hypothesized that CAF could trigger a signal cascade which culminates by changing the binding of transcription factors on the viral promoter, leading to a decrease in transcription efficiency.

Our results using the progressively deleted constructs of the HIV-1 LTR indicate that each deletion affects transcription of the LTR, because of the loss of various transcription factor binding sites (negative or positive regulators) and enhancers that are required for optimal transcription from the HIV-1 LTR. However, despite these differences in the level of transcription from these constructs, CAF was able to suppress transcription from all of the constructs. Data from these 5' LTR-deletion and TAR deletion constructs indicate that the HIV-1 minimal promoter consisting of the 3 SpI sites and TATA box is required for CAF mediated transcriptional suppression. Moreover, the requirement for 3SpI-TATA region for transcriptional suppression is confirmed by demonstrating transcriptional suppression of a promoter-less reporter gene construct carrying only these sites without TAR or any other upstream or downstream elements.

There are viral promoters that do not respond to CAF-mediated suppression of transcription. Among these is the early SV40 promoter, whose minimal promoter also consists of SpI sites, in addition to the TATA box (unpublished data from our lab). We attempted to insert the HIV-1 3SpI-TATA segment into the SV40 promoter to seek a conversion from CAF-resistance to CAF-susceptibility of the chimeric promoter. However, the chimeric promoter was transcriptionally inactive. Transcription from the SV40 promoter is known to be critically dependent upon the 6 SpI sites. Mutation of even one of the SpI sites in the minimal promoter region of SV40 is known to significantly decrease transcription. It is possible that the HIV SpI-TATA was not enough to compensate for the loss of the 6 SpI sites of SV40.

It is interesting to speculate about the various ways by which CAF might suppress transcription from the LTR region encompassing 3 SpI sites and TATA box. CAF has been shown to be equally effective against all HIV-1 clades. If its target on the LTR is indeed the minimal promoter, then it is likely that the sequence of the 3SpI-TATA segment is relatively conserved among all the clades. We aligned the sequences of the minimal promoter of HIV-1 Clades A1, A2, B, and C (Figure 34) and observed some differences in the SpI sequences between the different clades, although the sequence of the TATA box was conserved.

Figure 34: Alignment of minimal promoter region of HIV-1 Clades A1, A2, B, and C

The SpI sites (yellow highlights) and the TATA box (bold letters) of the LTR of single isolates of Clades A1, A2, and B and two isolates of clade C were aligned using ClustalW2. Ref refers to the sequence of the construct used in this study. A2 refers to the clade A2 isolate 94CY017.41 (Acc no. AF286237), B refers to the clade B HXB2 isolate (Acc no. K03455), A1 refers to the clade A1 isolate Q23-17 (Acc no. AF004885), $C_{(ETH)}$ refers to the clade C isolate ETH2220 (Acc no. U46016) and $C_{(IN)}$ refers to the clade C isolate IN21068 (Acc no. AF067155).

Not shown here are the sequences of the minimal promoters of HIV- 2 and SIV, against which CAF, from the CD8 cells of baboons and chimpanzees respectively, has shown to be active. Both HIV-2 and SIV contain 4 SpI sites upstream of the TATA box. The SpI sites of the

HIV-1 promoter are crucial for transcription. While SpI has been found to be important for the transcription of HIV-2 [247], it is thought to be dispensable for efficient transcription from the SIV promoter [248]. Yet, these sites do not just bind the SpI protein. These same sites act as recognition elements for other members of the Sp1 family, notably Sp3 and Sp4. While all members of the SpI family of proteins have similar structures and affinity for GC-rich sequences, their effects on transcription may be different. Sp4 has been shown to have activating effects on HIV transcription, similar to Sp1, whereas Sp3 competes with Sp1 for the same binding sites and represses transcription from the LTR [249]. It is possible that CAF, through the induction of some intermediate protein, promoters the binding of Sp3, over Sp1, to the HIV minimal promoter. Recent studies with chromatin immune-precipitation (ChIP) have shown that the proteins SpI, c-myc and HDAC1 form a complex at the HIV promoter, which represses transcription from the viral promoter and promotes latency [240]. The tumor suppresser and cell cycle protein p53 has been shown to bind SpI and repress transcription from the HIV LTR [250]. CAF might function by facilitating one of these processes.

CAF might also directly affect the TATA box, by adversely impacting one of the many proteins that constitute the transcription pre-initiation complex. The TATA box serves as the recognition element for the TATA box binding protein, TBP, which is a component of the general Transcription Factor TFIID. TFIID, through TBP, associates with the TATA box and in turn, recruits many other transcription initiation factors, known as TBP Associated Factors or TAFs to the site. Following the binding of TFII, TFIIB and RNA polymerase associate with the promoter, and then TFIIA, TFIIIE, and TFIIH follow. Thus, the initiation of transcription in

eukaryotic cells is a complex process and CAF might interrupt at any of these stages, leading to transcriptional repression.

Earlier studies to identify the region on the LTR necessary for CAF activity had suggested that multiple transcription factor binding sites may play a role in transcriptional suppression and had highlighted the importance of NFKB and the transcription factor NFAT/COUP-TF within the NRE in the suppressive process [251]. While our results provide further evidence for the multi-pronged nature of CAF action on the LTR, we found no requirement for NFKB or NFAT/COUP-TF for the action of CAF. To our knowledge, ours is the first study to identify the minimum target site on the LTR that is sufficient for transcriptional suppression.

Our studies show that the minimal region of the LTR that is necessary for CAF-mediated transcriptional suppression involves the 3 SpI sites and the TATA box, a region 90bp long. Complex and stochastic interactions between the proteins that bind this region can switch the balance between activation and inhibition of transcription, as other papers have shown [252-254]. A small change in the ratio of activating complexes, consisting of Histone Acetyltransferases (HATs), to repressing complexes, consisting of Histone Deacetylases (HDACs), can shift the balance from transcription activation to transcriptional suppression [252]. Thus, it is possible that CAF action on the target cell leads to protein modifications, such as deacetylation, on the LTR that tip the balance towards transcriptional suppression.

The story of CAF is intriguing because of its many implications. The study of transcriptional suppression by CAF may shed light on mechanisms of HIV-1 latency. HIV-1

LTR is associated with 5 precisely positioned nucleosomes, named nuc0 to nuc 4 [255]. It has been shown that one of these nucleosomes of the HIV-1 LTR is immediately downstream of the 3 SpI sites and TATA box, which are the targets of CAF. Latency of HIV-1 has been shown to be associated with the presence of deacetylated histones at these nucleosomes. If CAF suppresses gene expression from the LTR by recruiting histone deacetylases to this region, its mechanism of action may be similar to some of the events taking place during HIV-1 latency. The removal of CAF eventually leads to resurgence in viral replication. The events taking place at the LTR during this process might mirror those taking place during reactivation of virus after latency.

CAF is a host cellular factor that can suppress HIV-1 transcription. It is secreted by CD8 T cells, key players of the adaptive immune system, but does not appear to require either antigen presence [183, 256] or MHC restriction, displaying characteristics of an innate immune signaling protein. Hence, CAF might be a bridge between the adaptive and innate arms of the immune system. Activity of CAF has been found active against FIV and SIV, which infect cats and non-human primates respectively. Although there have been no studies looking at the effect of CAF on Equine Infectious Anemia virus (EIAV), the counterpart of HIV in horses, or on Caprine Arthritic Encephalitis Virus (CAEV), the counterpart of HIV in goats, it is possible that these animals have similar responses too. Perhaps CAF evolved as a mechanism in mammals to repress gene expression from retroviruses. Nearly 8% of the human genome is made of human retroviral elements [257]. A recent paper demonstrated that the expression of human endogenous retroviral (HERV) elements was no longer silenced in HIV-1 infected cells. The authors demonstrated that HIV-1 induced active transcription of genes from endogenous retroviral

promoters, leading not only to the production of HERV proteins, but also to the activation of HERV-specific CD8 T cells [258]. Certainly it is possible that there are host restriction factors which suppress not only the endogenous retroviral elements, but also invading retroviruses. There is already a precedence for a host restriction factor which has evolved to silence the expression from endogenous retroviral elements, while also displaying potent suppressive activities on HIV-1: APOBEC-3G, from humans, African Green Monkeys (AGM) and mice, has been shown to inhibit the gene transposition of two active murine retroviruses [259]. CAF might well be a second factor or a group of factors for the same purpose.

In conclusion, our results suggest that CAF acts by triggering a signal transduction cascade within the target cells, and acts on the 3 SpI-TATA segment of the HIV-1 LTR to effect suppression from the viral promoter.

8.0 FUTURE WORK

Determination of alterations to protein binding on the HIV LTR in response to CAF:

We have identified a minimal region on the HIV LTR that is necessary for CAF action. This region consists of the 3 SpI sites and TATA box. Using ChIP assays combined with Mass spectrometry, it will be possible to identify the exact proteins binding to this region, upon exposure to CAF. Once these proteins have been identified, their upstream binding partners and regulators of activation can be identified. Then these proteins, in turn, can be examined for their role in the CAF-mediated suppressive process.

Identification of signaling cascades in cells treated with CAF, by tracking phosphorylation patterns:

Another method to identify signaling cascades involved in the HIV transcriptional suppression process in cells treated with CAF is by mass spectrometry. Mass spectrometry of protein lysates from cells treated with CAF might be able to identify proteins that are being specifically phosphorylated in response to the treatment. Phosphorylation is probably the most common method of intracellular signal transduction. Hence it is possible that CAF activates some phosphorylation cascades within the target cell, even though our method using antibody coated beads could not detect these. Once a list of these proteins is obtained, they will then be examined for their role in the transcription suppression process.

Identification of "CAF-Resistant" cell lines to determine proteins that confer resistance/susceptibility to CAF: it is possible that there are cell lines in which HIV transcription does not get suppressed in response to CAF. In fact, TZM-bl cells, themselves, might be a possible candidate for this phenotype, since they show no suppressive ability when treated with IFNγ-depleted cultures. Once a cell line is identified that consistently shows no suppression in response to CAF treatments, gene transfer experiments may be performed to identify specific genes or gene products that are necessary for conferring susceptibility to CAF.

The relationship between Regulatory T cells and CAF production by CD8 T cells as a possible method to identify CAF : CAF activity has been shown to correlate with clinical status of disease. For instance, CD8 cells from patients who are in the acute stage of infection are better able to suppress HIV transcription than those from patients who are in the stage of AIDS. While it is understandable that CD8 cells from patients who are at a more advanced stage of disease are likely to be dysfunctional due to a variety of reasons (some of which are detailed in the Introduction section), perhaps it is possible that the state of activation of the cell also has something to do with the ability to mediate viral suppression by CAF. Many studies have shown that non-pathogenic SIV infection in AGMs, Sooty Mangabeys and chimpanzees is associated with a lack of hyperactivation of the immune system, in contrast to what is seen in humans or rhesus macaques. Indeed, chronic activation of the immune system appears to be one of the crucial prognostic markers for disease progression in humans.

Regulatory T cells have been shown in asymptomatic, healthy HIV-1 infected individuals to significantly lower plasma viremia, play a role in maintaining high CD4+:CD8+ T cell ratios and are favorable clinical markers of disease status. In vitro, they are known to suppress cellular

proliferation and cytokine production by CD4 and CD8 T cells [260]. Treg depletion has also been known to be associated with higher levels of cellular activation in infected subjects [261].

It would be interesting to determine if CD8 cells from patients in the chronic stage of HIV regain CAF activity in vitro if the cells were co-cultured in the presence of regulatory T cells. If they do, further experiments could be performed to find out which genes, activated in these CD8 T cells in response to the Tregs, are responsible for the production of CAF. These experiments might take us closer to finding out the identity of CAF, and indeed, if CAF is a multi-factorial or a single protein.

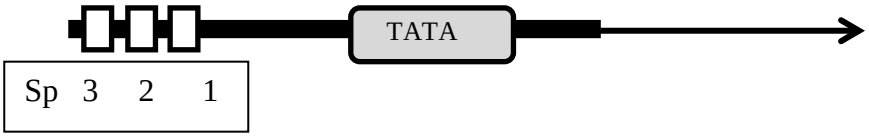
APPENDIX

LIST OF DNA OLIGONUCLEOTIDES AND PRIMERS TO CREATE SP1 DELETION MUTATIONS AND INACTIVATING POINT MUTATIONS

SpI deletion construct oligos and primers

No	Name of construct	DNA oligos used	PCR primers
1	Del 1, del 2	5’TCGGAGGACAGTACTCCGACCCGGTCGAAG GGAGGCGTGGCCTGAGCCCTCAGATCCTGCA**T ATAA**GC And 5’GAGCCCTCAGATCCTGCA**TATAA**GCAGCTG CTTTTTGCCTGTACTGGGTCTCTCTGGTTAGAC CAGATCCGAGC	Fwd:TGTGAAGCTTTCGGAGG ACAGTACTC Rev:TGTGGAGCTCGGATCTG GTCTAAC
2	Del 1	5’TCGGAGGACAGTACTCCGACCCGGTCGAAG GGAGGCGTGGCCTGGGCGGGACTGAGCCCTC AGATCCTGCA**TATAA**GC And 5’GAGCCCTCAGATCCTGCA**TATAA**GCAGCTG CTTTTTGCCTGTACTGGGTCTCTCTGGTTAGAC CAGATCCGAGC	Same as above
3	Del 2	5’TCGGAGGACAGTACTCCGACCCGGTCGAAG GGAGGCGTGGCCTTGGGGAGTGGCGAGCCCT CAGATCCTGCA**TATAA** And 5’GAGCCCTCAGATCCTGCA**TATAA**GCAGCTG CTTTTTGCCTGTACTGGGTCTCTCTGGTTAGAC CAGATCCGAGC	Same as above

SpI inactivating point mutation oligos and primers



Name of construct	DNA Oligos used	Primers used
Mut 1	Oligo 1: TCGGAGGACAGTACTCCGACCCGGTCGAAGGGAGGCGTGGCCTG GGCGGGACTGGTTAGTGGCGAGCCCTCAGATCCTGCA**TATAA** Oligo 2: GAGCCCTCAGATCCTGCA**TATAA**GCAGCTGCTTTTTGCCTGTACT GGGTCTCTCTGGTTAGACCAGATCCGAGC	Fwd:TGTGAAGCTTTC GGAGGACAGTACTC Rev:TGTGGAGCTCGG ATCTGGTCTAAC
Mut 2	Oligo 1: TCGGAGGACAGTACTCCGACCCGGTCGAAGGGAGGCGTGGCCTG TTCGGGACTGGGGAGTGGCGAGCCCTCAGATCCTGCA**TATAA** Oligo 2: same as above	Same as above
Mut 3	Oligo 1: TCGGAGGACAGTACTCCGACCCGGTCGAAGGGATTCGTGGCCTG GGCGGGACTGGGGAGTGGCGAGCCCTCAGATCCTGCA**TATAA** Oligo 2: same as above	Same as above
Mut 1, 2	Oligo 1: TCGGAGGACAGTACTCCGACCCGGTCGAAGGGAGGCGTGGCCTG TTCGGGACTGGTTAGTGGCGAGCCCTCAGATCCTGCA**TATAA** Oligo 2: same as above	Same as above
Mut 1, 3	Oligo 1: TCGGAGGACAGTACTCCGACCCGGTCGAAGGGATTCGTGGCCTG GGCGGGACTGGTTAGTGGCGAGCCCTCAGATCCTGCA**TATAA** Oligo 2: same as above	Same as above
Mut 2, 3	Oligo 1: TCGGAGGACAGTACTCCGACCCGGTCGAAGGGATTCGTGGCCTG TTCGGGACTGGGGAGTGGCGAGCCCTCAGATCCTGCA**TATAA** Oligo 2: same as above	Same as above

BIBLIOGRAPHY

1. Joint United Nations Programme on HIV/AIDS, W.H.O., *AIDS Epidemic Update: December 2009*. 2009. p. http://www.who.int/hiv/pub/epidemiology/epidemic/en/

2. Hutchinson, A.B., Farnham, P.G., Dean, H.D., Ekwueme, D.U., del Rio, C., Kamimoto, L., Kellerman, S.E., *The economic burden of HIV in the United States in the era of highly active antiretroviral therapy: evidence of continuing racial and ethnic differences.* J Acquir Immune Defic Syndr, 2006. **43**(4): p. 451-7.
3. Permanyer, M., E. Ballana, and J.A. Este, *Endocytosis of HIV: anything goes.* Trends Microbiol. **18**(12): p. 543-51.
4. Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, A.M., *Principles of Virology: Molecular Biology, Pathogenesis and the Control of Animal Viruses*. 2nd ed. 2004: ASM.
5. Miyauchi, K., et al., *HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes.* Cell, 2009. **137**(3): p. 433-44.
6. Mitchell, R.S., et al., *Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences.* PLoS Biol, 2004. **2**(8): p. E234.
7. Wang, G.P., et al., *HIV integration site selection: analysis by massively parallel pyrosequencing reveals association with epigenetic modifications.* Genome Res, 2007. **17**(8): p. 1186-94.
8. Debyser, Z. and F. Christ, *On the cell biology of HIV integration from basic research to development of novel antiviral drugs.* Verh K Acad Geneeskd Belg. **72**(5-6): p. 219-37.
9. Ono, A., et al., *Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane.* Proc Natl Acad Sci U S A, 2004. **101**(41): p. 14889-94.
10. Saad, J.S., et al., *Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly.* Proc Natl Acad Sci U S A, 2006. **103**(30): p. 11364-9.
11. Bieniasz, P.D., *The cell biology of HIV-1 virion genesis.* Cell Host Microbe, 2009. **5**(6): p. 550-8.
12. D'Souza, V. and M.F. Summers, *How retroviruses select their genomes.* Nat Rev Microbiol, 2005. **3**(8): p. 643-55.
13. Mehandru, S., et al., *Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract.* J Exp Med, 2004. **200**(6): p. 761-70.
14. Li, Q., et al., *Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells.* Nature, 2005. **434**(7037): p. 1148-52.
15. Brenchley, J.M., D.A. Price, and D.C. Douek, *HIV disease: fallout from a mucosal catastrophe?* Nat Immunol, 2006. **7**(3): p. 235-9.

16. Mattapallil, J.J., et al., *Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection.* Nature, 2005. **434**(7037): p. 1093-7.
17. Koup, R.A., et al., *Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.* J Virol, 1994. **68**(7): p. 4650-5.
18. Borrow, P., et al., *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.* J Virol, 1994. **68**(9): p. 6103-10.
19. Price, D.A., et al., *Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection.* Proc Natl Acad Sci U S A, 1997. **94**(5): p. 1890-5.
20. Borrow, P., et al., *Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus.* Nat Med, 1997. **3**(2): p. 205-11.
21. Walker, C.M., et al., *CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication.* Science, 1986. **234**(4783): p. 1563-6.
22. Streeck, H., et al., *Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells.* J Virol, 2009. **83**(15): p. 7641-8.
23. Douek, D.C., L.J. Picker, and R.A. Koup, *T cell dynamics in HIV-1 infection.* Annu Rev Immunol, 2003. **21**: p. 265-304.
24. Brenchley, J.M., et al., *Microbial translocation is a cause of systemic immune activation in chronic HIV infection.* Nat Med, 2006. **12**(12): p. 1365-71.
25. Brenchley, J.M., et al., *Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections.* Blood, 2008. **112**(7): p. 2826-35.
26. Deeks, S.G., et al., *Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load.* Blood, 2004. **104**(4): p. 942-7.
27. Gordon, S.N., et al., *Severe depletion of mucosal CD4+ T cells in AIDS-free simian immunodeficiency virus-infected sooty mangabeys.* J Immunol, 2007. **179**(5): p. 3026-34.
28. Hazenberg, M.D., et al., *Depletion of naive CD4 T cells by CXCR4-using HIV-1 variants occurs mainly through increased T-cell death and activation.* AIDS, 2003. **17**(10): p. 1419-24.
29. Hazenberg, M.D., et al., *Persistent immune activation in HIV-1 infection is associated with progression to AIDS.* AIDS, 2003. **17**(13): p. 1881-8.
30. Pandrea, I.V., et al., *Acute loss of intestinal CD4+ T cells is not predictive of simian immunodeficiency virus virulence.* J Immunol, 2007. **179**(5): p. 3035-46.
31. Sousa, A.E., et al., *CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load.* J Immunol, 2002. **169**(6): p. 3400-6.
32. Papagno, L., et al., *Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection.* PLoS Biol, 2004. **2**(2): p. E20.
33. Lewis, D.E., et al., *Anergy and apoptosis in CD8+ T cells from HIV-infected persons.* J Immunol, 1994. **153**(1): p. 412-20.
34. Day, C.L., et al., *PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.* Nature, 2006. **443**(7109): p. 350-4.

35. Moir, S. and A.S. Fauci, *B cells in HIV infection and disease.* Nat Rev Immunol, 2009. **9**(4): p. 235-45.
36. Herbeuval, J.P., et al., *TNF-related apoptosis-inducing ligand (TRAIL) in HIV-1-infected patients and its in vitro production by antigen-presenting cells.* Blood, 2005. **105**(6): p. 2458-64.
37. Kuerten, S., et al., *The TRAIL of helpless CD8+ T cells in HIV infection.* AIDS Res Hum Retroviruses, 2008. **24**(9): p. 1175-83.
38. Streeck, H. and D.F. Nixon, *T cell immunity in acute HIV-1 infection.* J Infect Dis. **202 Suppl 2**: p. S302-8.
39. Chu, C. and P.A. Selwyn, *Complications of HIV infection: a systems-based approach.* Am Fam Physician, 2011. **83**(4): p. 395-406.
40. Doitsh, G., et al., *Abortive HIV infection mediates CD4 T cell depletion and inflammation in human lymphoid tissue.* Cell. **143**(5): p. 789-801.
41. Fevrier, M., Dorgham, K., Rebollo, A., *CD4+ T Cell Depletion in Human Immunodeficiency Virus (HIV) Infection: Role of Apoptosis.* Viruses, 2011. **3**: p. 586-612.
42. Douek, D.C., et al., *HIV preferentially infects HIV-specific CD4+ T cells.* Nature, 2002. **417**(6884): p. 95-8.
43. Collins, K.L., et al., *HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes.* Nature, 1998. **391**(6665): p. 397-401.
44. Li, C.J., et al., *Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein.* Science, 1995. **268**(5209): p. 429-31.
45. Grossman, Z., et al., *Pathogenesis of HIV infection: what the virus spares is as important as what it destroys.* Nat Med, 2006. **12**(3): p. 289-95.
46. He, H., et al., *Functional Impairment of Central Memory CD4 T Cells Is a Potential Early Prognostic Marker for Changing Viral Load in SHIV-Infected Rhesus Macaques.* PLoS One. **6**(5): p. e19607.
47. Okoye, A., et al., *Progressive CD4+ central memory T cell decline results in CD4+ effector memory insufficiency and overt disease in chronic SIV infection.* J Exp Med, 2007. **204**(9): p. 2171-85.
48. Holmes, D., et al., *Foxp3 and Treg cells in HIV-1 infection and immuno-pathogenesis.* Immunol Res, 2008. **41**(3): p. 248-66.
49. McKenna, K., A.S. Beignon, and N. Bhardwaj, *Plasmacytoid dendritic cells: linking innate and adaptive immunity.* J Virol, 2005. **79**(1): p. 17-27.
50. Biron, C.A. and R.T. Gazzinelli, *Effects of IL-12 on immune responses to microbial infections: a key mediator in regulating disease outcome.* Curr Opin Immunol, 1995. **7**(4): p. 485-96.
51. Kubin, M., J.M. Chow, and G. Trinchieri, *Differential regulation of interleukin-12 (IL-12), tumor necrosis factor alpha, and IL-1 beta production in human myeloid leukemia cell lines and peripheral blood mononuclear cells.* Blood, 1994. **83**(7): p. 1847-55.
52. Trinchieri, G., *Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes.* Blood, 1994. **84**(12): p. 4008-27.
53. Wolf, S.F., D. Sieburth, and J. Sypek, *Interleukin 12: a key modulator of immune function.* Stem Cells, 1994. **12**(2): p. 154-68.
54. Colonna, M., G. Trinchieri, and Y.J. Liu, *Plasmacytoid dendritic cells in immunity.* Nat Immunol, 2004. **5**(12): p. 1219-26.

55. Altfeld, M., et al., *DCs and NK cells: critical effectors in the immune response to HIV-1.* Nat Rev Immunol. **11**(3): p. 176-86.
56. Ferlazzo, G., et al., *Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs.* Proc Natl Acad Sci U S A, 2004. **101**(47): p. 16606-11.
57. Lanzavecchia, A. and F. Sallusto, *Regulation of T cell immunity by dendritic cells.* Cell, 2001. **106**(3): p. 263-6.
58. Barron, M.A., et al., *Influence of plasma viremia on defects in number and immunophenotype of blood dendritic cell subsets in human immunodeficiency virus 1-infected individuals.* J Infect Dis, 2003. **187**(1): p. 26-37.
59. Donaghy, H., et al., *Loss of blood CD11c(+) myeloid and CD11c(-) plasmacytoid dendritic cells in patients with HIV-1 infection correlates with HIV-1 RNA virus load.* Blood, 2001. **98**(8): p. 2574-6.
60. Meyers, J.H., et al., *Impact of HIV on cell survival and antiviral activity of plasmacytoid dendritic cells.* PLoS One, 2007. **2**(5): p. e458.
61. Feldman, S., et al., *Decreased interferon-alpha production in HIV-infected patients correlates with numerical and functional deficiencies in circulating type 2 dendritic cell precursors.* Clin Immunol, 2001. **101**(2): p. 201-10.
62. Kamga, I., et al., *Type I interferon production is profoundly and transiently impaired in primary HIV-1 infection.* J Infect Dis, 2005. **192**(2): p. 303-10.
63. Chehimi, J., et al., *Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals.* J Immunol, 2002. **168**(9): p. 4796-801.
64. Hsieh, S.M., et al., *Differential impact of late-stage HIV-1 infection on in vitro and in vivo maturation of myeloid dendritic cells.* J Acquir Immune Defic Syndr, 2003. **33**(4): p. 413-9.
65. Kawamura, T., et al., *Decreased stimulation of CD4+ T cell proliferation and IL-2 production by highly enriched populations of HIV-infected dendritic cells.* J Immunol, 2003. **170**(8): p. 4260-6.
66. Lore, K., et al., *Accumulation of DC-SIGN+CD40+ dendritic cells with reduced CD80 and CD86 expression in lymphoid tissue during acute HIV-1 infection.* AIDS, 2002. **16**(5): p. 683-92.
67. Granelli-Piperno, A., et al., *HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation.* Proc Natl Acad Sci U S A, 2004. **101**(20): p. 7669-74.
68. Patterson, S., et al., *Human BDCA-1-positive blood dendritic cells differentiate into phenotypically distinct immature and mature populations in the absence of exogenous maturational stimuli: differentiation failure in HIV infection.* J Immunol, 2005. **174**(12): p. 8200-9.
69. Arrighi, J.F., et al., *DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells.* J Exp Med, 2004. **200**(10): p. 1279-88.
70. McDonald, D., et al., *Recruitment of HIV and its receptors to dendritic cell-T cell junctions.* Science, 2003. **300**(5623): p. 1295-7.
71. Wiley, R.D. and S. Gummuluru, *Immature dendritic cell-derived exosomes can mediate HIV-1 trans infection.* Proc Natl Acad Sci U S A, 2006. **103**(3): p. 738-43.

72. Kawamura, T., et al., *R5 HIV productively infects Langerhans cells, and infection levels are regulated by compound CCR5 polymorphisms.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8401-6.
73. Lore, K., et al., *Myeloid and plasmacytoid dendritic cells transfer HIV-1 preferentially to antigen-specific CD4+ T cells.* J Exp Med, 2005. **201**(12): p. 2023-33.
74. Nobile, C., et al., *Covert human immunodeficiency virus replication in dendritic cells and in DC-SIGN-expressing cells promotes long-term transmission to lymphocytes.* J Virol, 2005. **79**(9): p. 5386-99.
75. Venkatachari, N.J., et al., *HIV-1 infection of DC: evidence for the acquisition of virus particles from infected T cells by antigen uptake mechanism.* PLoS One, 2009. **4**(10): p. e7470.
76. McElrath, M.J., J.E. Pruett, and Z.A. Cohn, *Mononuclear phagocytes of blood and bone marrow: comparative roles as viral reservoirs in human immunodeficiency virus type 1 infections.* Proc Natl Acad Sci U S A, 1989. **86**(2): p. 675-9.
77. Orenstein, J.M., C. Fox, and S.M. Wahl, *Macrophages as a source of HIV during opportunistic infections.* Science, 1997. **276**(5320): p. 1857-61.
78. Schuitemaker, H., et al., *Proliferation-dependent HIV-1 infection of monocytes occurs during differentiation into macrophages.* J Clin Invest, 1992. **89**(4): p. 1154-60.
79. Collman, R., et al., *Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). Monocyte-tropic and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types.* J Exp Med, 1989. **170**(4): p. 1149-63.
80. Coleman, C.M. and L. Wu, *HIV interactions with monocytes and dendritic cells: viral latency and reservoirs.* Retrovirology, 2009. **6**: p. 51.
81. Fantuzzi, L., F. Belardelli, and S. Gessani, *Monocyte/macrophage-derived CC chemokines and their modulation by HIV-1 and cytokines: a complex network of interactions influencing viral replication and AIDS pathogenesis.* J Leukoc Biol, 2003. **74**(5): p. 719-25.
82. Kedzierska, K., et al., *Defective phagocytosis by human monocyte/macrophages following HIV-1 infection: underlying mechanisms and modulation by adjunctive cytokine therapy.* J Clin Virol, 2003. **26**(2): p. 247-63.
83. Kottilil, S., et al., *Innate immune dysfunction in HIV infection: effect of HIV envelope-NK cell interactions.* J Immunol, 2006. **176**(2): p. 1107-14.
84. Leibson, P.J., *Cytotoxic lymphocyte recognition of HLA-E: utilizing a nonclassical window to peer into classical MHC.* Immunity, 1998. **9**(3): p. 289-94.
85. Tay, C.H., E. Szomolanyi-Tsuda, and R.M. Welsh, *Control of infections by NK cells.* Curr Top Microbiol Immunol, 1998. **230**: p. 193-220.
86. Yokoyama, W.M., *Natural killer cell receptors.* Curr Opin Immunol, 1998. **10**(3): p. 298-305.
87. Cohen, G.B., et al., *The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells.* Immunity, 1999. **10**(6): p. 661-71.
88. Mavilio, D., et al., *Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 15011-6.

89. Zocchi, M.R., et al., *HIV-1 Tat inhibits human natural killer cell function by blocking L-type calcium channels.* J Immunol, 1998. **161**(6): p. 2938-43.
90. Ahmad, A. and J. Menezes, *Defective killing activity against gp120/41-expressing human erythroleukaemic K562 cell line by monocytes and natural killer cells from HIV-infected individuals.* AIDS, 1996. **10**(2): p. 143-9.
91. Majumder, B., et al., *Infection with Vpr-positive human immunodeficiency virus type 1 impairs NK cell function indirectly through cytokine dysregulation of infected target cells.* J Virol, 2008. **82**(14): p. 7189-200.
92. Tiemessen, M.M., et al., *Transforming growth factor-beta inhibits human antigen-specific CD4+ T cell proliferation without modulating the cytokine response.* Int Immunol, 2003. **15**(12): p. 1495-504.
93. Chung, A.W., et al., *Activation of NK cells by ADCC antibodies and HIV disease progression.* J Acquir Immune Defic Syndr, 2011.
94. Tomaras, G.D., et al., *Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia.* J Virol, 2008. **82**(24): p. 12449-63.
95. Frost, S.D., et al., *Antibody responses in primary HIV-1 infection.* Curr Opin HIV AIDS, 2008. **3**(1): p. 45-51.
96. Richman, D.D., et al., *Rapid evolution of the neutralizing antibody response to HIV type 1 infection.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4144-9.
97. Gray, E.S., et al., *Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection.* J Virol, 2007. **81**(12): p. 6187-96.
98. Shen, X., et al., *In vivo gp41 antibodies targeting the 2F5 monoclonal antibody epitope mediate human immunodeficiency virus type 1 neutralization breadth.* J Virol, 2009. **83**(8): p. 3617-25.
99. Stamatatos, L., et al., *Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine?* Nat Med, 2009. **15**(8): p. 866-70.
100. Haynes, B.F. and D.C. Montefiori, *Aiming to induce broadly reactive neutralizing antibody responses with HIV-1 vaccine candidates.* Expert Rev Vaccines, 2006. **5**(3): p. 347-63.
101. Pantophlet, R. and D.R. Burton, *GP120: target for neutralizing HIV-1 antibodies.* Annu Rev Immunol, 2006. **24**: p. 739-69.
102. Phogat, S., R.T. Wyatt, and G.B. Karlsson Hedestam, *Inhibition of HIV-1 entry by antibodies: potential viral and cellular targets.* J Intern Med, 2007. **262**(1): p. 26-43.
103. Cole, K.S., et al., *Evolution of envelope-specific antibody responses in monkeys experimentally infected or immunized with simian immunodeficiency virus and its association with the development of protective immunity.* J Virol, 1997. **71**(7): p. 5069-79.
104. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.* N Engl J Med, 2009. **361**(23): p. 2209-20.
105. Devash, Y., et al., *Vertical transmission of human immunodeficiency virus is correlated with the absence of high-affinity/avidity maternal antibodies to the gp120 principal neutralizing domain.* Proc Natl Acad Sci U S A, 1990. **87**(9): p. 3445-9.

106. Martinez-Maza, O., et al., *Infection with the human immunodeficiency virus (HIV) is associated with an in vivo increase in B lymphocyte activation and immaturity.* J Immunol, 1987. **138**(11): p. 3720-4.
107. Ammann, A.J., et al., *Acquired immune dysfunction in homosexual men: immunologic profiles.* Clin Immunol Immunopathol, 1983. **27**(3): p. 315-25.
108. Kopelman, R.G. and S. Zolla-Pazner, *Association of human immunodeficiency virus infection and autoimmune phenomena.* Am J Med, 1988. **84**(1): p. 82-8.
109. Macchia, D., et al., *Membrane tumour necrosis factor-alpha is involved in the polyclonal B-cell activation induced by HIV-infected human T cells.* Nature, 1993. **363**(6428): p. 464-6.
110. Chirmule, N., et al., *Nef protein of HIV-1 has B-cell stimulatory activity.* AIDS, 1994. **8**(6): p. 733-4.
111. Xu, W., et al., *HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via long-range intercellular conduits.* Nat Immunol, 2009. **10**(9): p. 1008-17.
112. Moir, S., et al., *B cells of HIV-1-infected patients bind virions through CD21-complement interactions and transmit infectious virus to activated T cells.* J Exp Med, 2000. **192**(5): p. 637-46.
113. Moir, S., et al., *Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals.* J Exp Med, 2008. **205**(8): p. 1797-805.
114. Jin, X., et al., *Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques.* J Exp Med, 1999. **189**(6): p. 991-8.
115. Schmitz, J.E., et al., *Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes.* Science, 1999. **283**(5403): p. 857-60.
116. Mellors, J.W., et al., *Prognostic value of HIV-1 RNA, CD4 cell count, and CD4 Cell count slope for progression to AIDS and death in untreated HIV-1 infection.* JAMA, 2007. **297**(21): p. 2349-50.
117. Mellors, J.W., et al., *Prognosis in HIV-1 infection predicted by the quantity of virus in plasma.* Science, 1996. **272**(5265): p. 1167-70.
118. Trautmann, L., et al., *Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction.* Nat Med, 2006. **12**(10): p. 1198-202.
119. Rocha, B. and C. Tanchot, *Towards a cellular definition of CD8+ T-cell memory: the role of CD4+ T-cell help in CD8+ T-cell responses.* Curr Opin Immunol, 2004. **16**(3): p. 259-63.
120. Shedlock, D.J. and H. Shen, *Requirement for CD4 T cell help in generating functional CD8 T cell memory.* Science, 2003. **300**(5617): p. 337-9.
121. Bourgeois, C., et al., *CD8 lethargy in the absence of CD4 help.* Eur J Immunol, 2002. **32**(8): p. 2199-207.
122. Janssen, E.M., et al., *CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death.* Nature, 2005. **434**(7029): p. 88-93.
123. Appay, V., et al., *HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function.* J Exp Med, 2000. **192**(1): p. 63-75.
124. Andersson, J., et al., *Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection.* AIDS, 1999. **13**(11): p. 1295-303.

125. Champagne, P., et al., *Skewed maturation of memory HIV-specific CD8 T lymphocytes.* Nature, 2001. **410**(6824): p. 106-11.
126. Carrington, M., et al., *HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage.* Science, 1999. **283**(5408): p. 1748-52.
127. Keet, I.P., et al., *Consistent associations of HLA class I and II and transporter gene products with progression of human immunodeficiency virus type 1 infection in homosexual men.* J Infect Dis, 1999. **180**(2): p. 299-309.
128. Trachtenberg, E., et al., *Advantage of rare HLA supertype in HIV disease progression.* Nat Med, 2003. **9**(7): p. 928-35.
129. Almeida, J.R., et al., *Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover.* J Exp Med, 2007. **204**(10): p. 2473-85.
130. Cocchi, F., et al., *Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells.* Science, 1995. **270**(5243): p. 1811-5.
131. Shaheen, F.a.C.R.G., *Co-receptor antagonists as HIV-1 entry inhibitors.* Curr Opin Infect Dis., 2004. **17**(1): p. 7-16.
132. Mosoian, A., et al., *Novel function of prothymosin alpha as a potent inhibitor of human immunodeficiency virus type 1 gene expression in primary macrophages.* J Virol, 2006. **80**(18): p. 9200-6.
133. Castelli, J., Thomas, E.K., Gilliet, M., Liu, Y-J., and Levy, J.A., *Mature dendritic cells can enhance CD8+ cell noncytotoxic anti-HIV responses: the role of IL15.* Blood, 2004. **103**: p. 2699-2704.
134. Walker, C.M. and J.A. Levy, *A diffusible lymphokine produced by CD8+ T lymphocytes suppresses HIV replication.* Immunology, 1989. **66**(4): p. 628-30.
135. Tumne, A., et al., *Noncytotoxic suppression of human immunodeficiency virus type 1 transcription by exosomes secreted from CD8+ T cells.* J Virol, 2009. **83**(9): p. 4354-64.
136. Ohashi, T., et al., *Role of class I major histocompatibility complex-restricted and -unrestricted suppression of human immunodeficiency virus type 1 replication by CD8+ T lymphocytes.* J Gen Virol, 1999. **80 (Pt 1)**: p. 209-16.
137. Levy, J.A., C.E. Mackewicz, and E. Barker, *Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8+ T cells.* Immunol Today, 1996. **17**(5): p. 217-24.
138. Mackewicz, C.E., H. Ortega, and J.A. Levy, *Effect of cytokines on HIV replication in CD4+ lymphocytes: lack of identity with the CD8+ cell antiviral factor.* Cell Immunol, 1994. **153**(2): p. 329-43.
139. Brinchmann, J.E., G. Gaudernack, and F. Vartdal, *CD8+ T cells inhibit HIV replication in naturally infected CD4+ T cells. Evidence for a soluble inhibitor.* J Immunol, 1990. **144**(8): p. 2961-6.
140. Mackewicz, C.E., E. Barker, and J.A. Levy, *Role of beta-chemokines in suppressing HIV replication.* Science, 1996. **274**(5291): p. 1393-5.
141. Mackewicz, C.E., et al., *Role of IL-16 in HIV replication.* Nature, 1996. **383**(6600): p. 488-9.
142. Mackewicz, C.E., et al., *HIV virions and HIV infection in vitro are unaffected by human granzymes A and B.* AIDS Res Hum Retroviruses, 2000. **16**(4): p. 367-72.

143. Mackewicz, C.E., et al., *Lack of the CD8+ cell anti-HIV factor in CD8+ cell granules.* Blood, 2003. **102**(1): p. 180-3.
144. Moriuchi, H., et al., *CD8+ T-cell-derived soluble factor(s), but not beta-chemokines RANTES, MIP-1 alpha, and MIP-1 beta, suppress HIV-1 replication in monocyte/macrophages.* Proc Natl Acad Sci U S A, 1996. **93**(26): p. 15341-5.
145. Lacey, S.F., et al., *The CXC chemokine stromal cell-derived factor 1 is not responsible for CD8+ T cell suppression of syncytia-inducing strains of HIV-1.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9842-7.
146. Chang, T.L., et al., *CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from alpha-defensin-1 HIV inhibition.* J Virol, 2003. **77**(12): p. 6777-84.
147. Levy, J.A., *The search for the CD8+ cell anti-HIV factor (CAF).* Trends Immunol, 2003. **24**(12): p. 628-32.
148. Gomez, A.M., F.M. Smaill, and K.L. Rosenthal, *Inhibition of HIV replication by CD8+ T cells correlates with CD4 counts and clinical stage of disease.* Clin Exp Immunol, 1994. **97**(1): p. 68-75.
149. Levy, J.A., et al., *CD8 cell noncytotoxic antiviral activity in human immunodeficiency virus-infected and -uninfected children.* J Infect Dis, 1998. **177**(2): p. 470-2.
150. Pollack, H., et al., *CD8+ T-cell-mediated suppression of HIV replication in the first year of life: association with lower viral load and favorable early survival.* AIDS, 1997. **11**(1): p. F9-13.
151. Dioszeghy, V., et al., *Changes in soluble factor-mediated CD8+ cell-derived antiviral activity in cynomolgus macaques infected with simian immunodeficiency virus SIVmac251: relationship to biological markers of progression.* J Virol, 2006. **80**(1): p. 236-45.
152. Blackbourn, D.J., et al., *Suppression of HIV replication by lymphoid tissue CD8+ cells correlates with the clinical state of HIV-infected individuals.* Proc Natl Acad Sci U S A, 1996. **93**(23): p. 13125-30.
153. Blackbourn, D.J., et al., *CD8+ cells from HIV-2-infected baboons control HIV replication.* AIDS, 1997. **11**(6): p. 737-46.
154. Castro, B.A., et al., *Suppression of human immunodeficiency virus replication by CD8+ cells from infected and uninfected chimpanzees.* Cell Immunol, 1991. **132**(1): p. 246-55.
155. Geiben-Lynn, R., et al., *Noncytolytic inhibition of X4 virus by bulk CD8(+) cells from human immunodeficiency virus type 1 (HIV-1)-infected persons and HIV-1-specific cytotoxic T lymphocytes is not mediated by beta-chemokines.* J Virol, 2001. **75**(17): p. 8306-16.
156. Kannagi, M., et al., *Suppression of simian immunodeficiency virus replication in vitro by CD8+ lymphocytes.* J Immunol, 1988. **140**(7): p. 2237-42.
157. Walker, C.M., et al., *CD8+ T cells from HIV-1-infected individuals inhibit acute infection by human and primate immunodeficiency viruses.* Cell Immunol, 1991. **137**(2): p. 420-8.
158. Jeng, C.R., et al., *Evidence for CD8+ antiviral activity in cats infected with feline immunodeficiency virus.* J Virol, 1996. **70**(4): p. 2474-80.
159. Hohdatsu, T., et al., *Studies on feline CD8+ T cell non-cytolytic anti-feline immunodeficiency virus (FIV) activity.* Arch Virol, 2000. **145**(12): p. 2525-38.

160. Landay, A.L., C.E. Mackewicz, and J.A. Levy, *An activated CD8+ T cell phenotype correlates with anti-HIV activity and asymptomatic clinical status.* Clin Immunol Immunopathol, 1993. **69**(1): p. 106-16.
161. Jiang, J.Q., et al., *Production of CD8+ T cell nonlytic suppressive factors by CD28, CD38, and HLA-DR subpopulations.* AIDS Res Hum Retroviruses, 2003. **19**(6): p. 497-502.
162. Biesinger, B., et al., *Stable growth transformation of human T lymphocytes by herpesvirus saimiri.* Proc Natl Acad Sci U S A, 1992. **89**(7): p. 3116-9.
163. Lacey, S.F., et al., *Herpesvirus saimiri transformation of HIV type 1 suppressive CD8+ lymphocytes from an HIV type 1-infected asymptomatic individual.* AIDS Res Hum Retroviruses, 1998. **14**(6): p. 521-31.
164. Mackewicz, C.E., et al., *Derivation of herpesvirus saimiri-transformed CD8+ T cell lines with noncytotoxic anti-HIV activity.* Clin Immunol Immunopathol, 1997. **82**(3): p. 274-81.
165. Chang, T.L., et al., *A soluble factor(s) secreted from CD8(+) T lymphocytes inhibits human immunodeficiency virus type 1 replication through STAT1 activation.* J Virol, 2002. **76**(2): p. 569-81.
166. Chen, Y., C. Rinaldo, and P. Gupta, *A semiquantitative assay for CD8+ T-cell-mediated suppression of human immunodeficiency virus type 1 infection.* Clin Diagn Lab Immunol, 1997. **4**(1): p. 4-10.
167. Chen, Y., et al., *Dependence of CD8+ T-cell-mediated suppression of HIV type 1 on viral phenotypes and mediation of phenotype-dependent suppression by viral envelope gene and not by beta-chemokines.* AIDS Res Hum Retroviruses, 2000. **16**(2): p. 117-24.
168. Southgate, C.D. and M.R. Green, *The HIV-1 Tat protein activates transcription from an upstream DNA-binding site: implications for Tat function.* Genes Dev, 1991. **5**(12B): p. 2496-507.
169. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.
170. Mackewicz, C.E., C.S. Craik, and J.A. Levy, *The CD8+ cell noncytotoxic anti-HIV response can be blocked by protease inhibitors.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3433-8.
171. Spang, A., *On the fate of early endosomes.* Biol Chem, 2009. **390**(8): p. 753-9.
172. Harding, C., J. Heuser, and P. Stahl, *Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes.* J Cell Biol, 1983. **97**(2): p. 329-39.
173. Fevrier, B. and G. Raposo, *Exosomes: endosomal-derived vesicles shipping extracellular messages.* Curr Opin Cell Biol, 2004. **16**(4): p. 415-21.
174. van Niel, G., et al., *Exosomes: a common pathway for a specialized function.* J Biochem, 2006. **140**(1): p. 13-21.
175. Thery, C., L. Zitvogel, and S. Amigorena, *Exosomes: composition, biogenesis and function.* Nat Rev Immunol, 2002. **2**(8): p. 569-79.
176. Mackewicz, C.E., M.R. Garovoy, and J.A. Levy, *HLA compatibility requirements for CD8(+)-T-cell-mediated suppression of human immunodeficiency virus replication.* J Virol, 1998. **72**(12): p. 10165-70.

177. Locher, C.P., D.J. Blackbourn, and J.A. Levy, *Suppression of human immunodeficiency virus type 1 replication by a soluble factor produced by CD8+ lymphocytes from HIV-2-infected baboons.* Immunol Lett, 1999. **66**(1-3): p. 151-7.
178. Mackewicz, C.E., H.W. Ortega, and J.A. Levy, *CD8+ cell anti-HIV activity correlates with the clinical state of the infected individual.* J Clin Invest, 1991. **87**(4): p. 1462-6.
179. Chen, C.H., et al., *CD8+ T lymphocyte-mediated inhibition of HIV-1 long terminal repeat transcription: a novel antiviral mechanism.* AIDS Res Hum Retroviruses, 1993. **9**(11): p. 1079-86.
180. Mackewicz, C.E., et al., *CD8(+) cell noncytotoxic anti-human immunodeficiency virus response inhibits expression of viral RNA but not reverse transcription or provirus integration.* J Gen Virol, 2000. **81**(Pt 5): p. 1261-4.
181. Overman, R.G., et al., *Initiation of human immunodeficiency virus type 1 (HIV-1) transcription is inhibited by noncytolytic CD8 suppression.* Open Virol J, 2007. **1**: p. 1-7.
182. Tomaras, G.D., et al., *CD8+ T cell-mediated suppressive activity inhibits HIV-1 after virus entry with kinetics indicating effects on virus gene expression.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3503-8.
183. Copeland, K.F., P.J. McKay, and K.L. Rosenthal, *Suppression of activation of the human immunodeficiency virus long terminal repeat by CD8+ T cells is not lentivirus specific.* AIDS Res Hum Retroviruses, 1995. **11**(11): p. 1321-6.
184. Saha, K., G. McKinley, and D.J. Volsky, *Improvement of Herpesvirus saimiri T cell immortalization procedure to generate multiple CD4+ T-cell clones from peripheral blood lymphocytes of AIDS patients.* J Immunol Methods, 1997. **206**(1-2): p. 21-3.
185. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.
186. Stephanou, A. and D.S. Latchman, *STAT-1: a novel regulator of apoptosis.* Int J Exp Pathol, 2003. **84**(6): p. 239-44.
187. Yang, J. and G.R. Stark, *Roles of unphosphorylated STATs in signaling.* Cell Res, 2008. **18**(4): p. 443-51.
188. Darnell, J.E., Jr., I.M. Kerr, and G.R. Stark, *Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins.* Science, 1994. **264**(5164): p. 1415-21.
189. Look, D.C., et al., *Stat1 depends on transcriptional synergy with Sp1.* J Biol Chem, 1995. **270**(51): p. 30264-7.
190. Kramer, O.H., et al., *Acetylation of Stat1 modulates NF-kappaB activity.* Genes Dev, 2006. **20**(4): p. 473-85.
191. Ouchi, T., et al., *Collaboration of signal transducer and activator of transcription 1 (STAT1) and BRCA1 in differential regulation of IFN-gamma target genes.* Proc Natl Acad Sci U S A, 2000. **97**(10): p. 5208-13.
192. Ramana, C.V., et al., *Regulation of c-myc expression by IFN-gamma through Stat1-dependent and -independent pathways.* EMBO J, 2000. **19**(2): p. 263-72.
193. Platanias, L.C., *Mechanisms of type-I- and type-II-interferon-mediated signalling.* Nat Rev Immunol, 2005. **5**(5): p. 375-86.
194. Emilie, D., et al., *Antagonistic effect of interferon-gamma on tat-induced transactivation of HIV long terminal repeat.* J Biol Chem, 1992. **267**(29): p. 20565-70.
195. Wong, G.H., et al., *In vitro anti-human immunodeficiency virus activities of tumor necrosis factor-alpha and interferon-gamma.* J Immunol, 1988. **140**(1): p. 120-4.

196. Pedroza-Martins, L., et al., *Interferon-gamma decreases replication of primary R5 HIV-1 isolates in thymocytes.* AIDS, 2006. **20**(6): p. 939-42.
197. Sarol, L.C., et al., *Inhibitory effects of IFN-gamma on HIV-1 replication in latently infected cells.* Biochem Biophys Res Commun, 2002. **291**(4): p. 890-6.
198. Vyakarnam, A., et al., *Tumour necrosis factors (alpha, beta) induced by HIV-1 in peripheral blood mononuclear cells potentiate virus replication.* AIDS, 1990. **4**(1): p. 21-7.
199. Yamamoto, J.K., et al., *Human alpha- and beta-interferon but not gamma- suppress the in vitro replication of LAV, HTLV-III, and ARV-2.* J Interferon Res, 1986. **6**(2): p. 143-52.
200. Chatterjee-Kishore, M., et al., *How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene.* EMBO J, 2000. **19**(15): p. 4111-22.
201. John, J., et al., *Isolation and characterization of a new mutant human cell line unresponsive to alpha and beta interferons.* Mol Cell Biol, 1991. **11**(8): p. 4189-95.
202. McKendry, R., et al., *High-frequency mutagenesis of human cells and characterization of a mutant unresponsive to both alpha and gamma interferons.* Proc Natl Acad Sci U S A, 1991. **88**(24): p. 11455-9.
203. Pellegrini, S., et al., *Use of a selectable marker regulated by alpha interferon to obtain mutations in the signaling pathway.* Mol Cell Biol, 1989. **9**(11): p. 4605-12.
204. Zarubin, T. and J. Han, *Activation and signaling of the p38 MAP kinase pathway.* Cell Res, 2005. **15**(1): p. 11-8.
205. Castelli, J., et al., *Mature dendritic cells can enhance CD8+ cell noncytotoxic anti-HIV responses: the role of IL-15.* Blood, 2004. **103**(7): p. 2699-704.
206. Cook, J.A., A. August, and A.J. Henderson, *Recruitment of phosphatidylinositol 3-kinase to CD28 inhibits HIV transcription by a Tat-dependent mechanism.* J Immunol, 2002. **169**(1): p. 254-60.
207. Horie, R., et al., *TRAF activation of C/EBPbeta (NF-IL6) via p38 MAPK induces HIV-1 gene expression in monocytes/macrophages.* Microbes Infect, 2007. **9**(6): p. 721-8.
208. Varin, A., et al., *Exogenous Nef protein activates NF-kappa B, AP-1, and c-Jun N-terminal kinase and stimulates HIV transcription in promonocytic cells. Role in AIDS pathogenesis.* J Biol Chem, 2003. **278**(4): p. 2219-27.
209. Tanaka, N., et al., *Interleukin-10 induces inhibitory C/EBPbeta through STAT-3 and represses HIV-1 transcription in macrophages.* Am J Respir Cell Mol Biol, 2005. **33**(4): p. 406-11.
210. Guo, B., et al., *Engagement of the human pre-B cell receptor generates a lipid raft-dependent calcium signaling complex.* Immunity, 2000. **13**(2): p. 243-53.
211. Papp, B. and R.A. Byrn, *Stimulation of HIV expression by intracellular calcium pump inhibition.* J Biol Chem, 1995. **270**(17): p. 10278-83.
212. Fluckiger, A.C., et al., *Btk/Tec kinases regulate sustained increases in intracellular Ca2+ following B-cell receptor activation.* EMBO J, 1998. **17**(7): p. 1973-85.
213. Dolfi, F., et al., *The adaptor protein Crk connects multiple cellular stimuli to the JNK signaling pathway.* Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15394-9.
214. Jones, K.A., et al., *Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1.* Science, 1986. **232**(4751): p. 755-9.
215. Kingsman, S.M. and A.J. Kingsman, *The regulation of human immunodeficiency virus type-1 gene expression.* Eur J Biochem, 1996. **240**(3): p. 491-507.

216. Nabel, G. and D. Baltimore, *An inducible transcription factor activates expression of human immunodeficiency virus in T cells.* Nature, 1987. **326**(6114): p. 711-3.
217. Cooney, A.J., et al., *Chicken ovalbumin upstream promoter transcription factor binds to a negative regulatory region in the human immunodeficiency virus type 1 long terminal repeat.* J Virol, 1991. **65**(6): p. 2853-60.
218. Lu, Y.C., et al., *Identification of cis-acting repressive sequences within the negative regulatory element of human immunodeficiency virus type 1.* J Virol, 1990. **64**(10): p. 5226-9.
219. Orchard, K., et al., *A novel T-cell protein which recognizes a palindromic sequence in the negative regulatory element of the human immunodeficiency virus long terminal repeat.* J Virol, 1990. **64**(7): p. 3234-9.
220. Fischer-Smith, T. and J. Rappaport, *Evolving paradigms in the pathogenesis of HIV-1-associated dementia.* Expert Rev Mol Med, 2005. **7**(27): p. 1-26.
221. Feng, S. and E.C. Holland, *HIV-1 tat trans-activation requires the loop sequence within tar.* Nature, 1988. **334**(6178): p. 165-7.
222. Wei, P., et al., *A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA.* Cell, 1998. **92**(4): p. 451-62.
223. Bonneau, K.R., et al., *Derivation of infectious HIV-1 molecular clones with LTR mutations: sensitivity to the CD8+ cell noncytotoxic anti-HIV response.* Virology, 2008. **373**(1): p. 30-8.
224. Perkins, N.D., et al., *A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation.* EMBO J, 1993. **12**(9): p. 3551-8.
225. Doetzlhofer, A., et al., *Histone deacetylase 1 can repress transcription by binding to Sp1.* Mol Cell Biol, 1999. **19**(8): p. 5504-11.
226. Suzuki, T., et al., *Regulation of interaction of the acetyltransferase region of p300 and the DNA-binding domain of Sp1 on and through DNA binding.* Genes Cells, 2000. **5**(1): p. 29-41.
227. Delling, U., et al., *Conserved nucleotides in the TAR RNA stem of human immunodeficiency virus type 1 are critical for Tat binding and trans activation: model for TAR RNA tertiary structure.* J Virol, 1992. **66**(5): p. 3018-25.
228. Berkhout, B. and K.T. Jeang, *Detailed mutational analysis of TAR RNA: critical spacing between the bulge and loop recognition domains.* Nucleic Acids Res, 1991. **19**(22): p. 6169-76.
229. Puglisi, J.D., et al., *Role of RNA structure in arginine recognition of TAR RNA.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3680-4.
230. Garcia, J.A., et al., *Human immunodeficiency virus type 1 LTR TATA and TAR region sequences required for transcriptional regulation.* EMBO J, 1989. **8**(3): p. 765-78.
231. Harrich, D., et al., *Role of SP1-binding domains in in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat.* J Virol, 1989. **63**(6): p. 2585-91.
232. Lillie, J.W. and M.R. Green, *Transcription activation by the adenovirus E1a protein.* Nature, 1989. **338**(6210): p. 39-44.
233. Lund, T., M.M. Medveczky, and P.G. Medveczky, *Herpesvirus saimiri Tip-484 membrane protein markedly increases p56lck activity in T cells.* J Virol, 1997. **71**(1): p. 378-82.

234. Weber, F., et al., *Transformation of human T-cell clones by Herpesvirus saimiri: intact antigen recognition by autonomously growing myelin basic protein-specific T cells.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 11049-53.
235. Yao, Z., et al., *Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor.* Immunity, 1995. **3**(6): p. 811-21.
236. Cazalla, D., T. Yario, and J.A. Steitz, *Down-regulation of a host microRNA by a Herpesvirus saimiri noncoding RNA.* Science. **328**(5985): p. 1563-6.
237. Lund, T.C., et al., *Activation of STAT transcription factors by herpesvirus Saimiri Tip-484 requires p56lck.* J Virol, 1997. **71**(9): p. 6677-82.
238. Brocke-Heidrich, K., et al., *BCL3 is induced by IL-6 via Stat3 binding to intronic enhancer HS4 and represses its own transcription.* Oncogene, 2006. **25**(55): p. 7297-304.
239. Mayer, K.D., et al., *Cutting edge: T-bet and IL-27R are critical for in vivo IFN-gamma production by CD8 T cells during infection.* J Immunol, 2008. **180**(2): p. 693-7.
240. Jiang, G., et al., *c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter.* J Virol, 2007. **81**(20): p. 10914-23.
241. Geonnotti, A.R., et al., *Differential inhibition of human immunodeficiency virus type 1 in peripheral blood mononuclear cells and TZM-bl cells by endotoxin-mediated chemokine and gamma interferon production.* AIDS Res Hum Retroviruses. **26**(3): p. 279-91.
242. Garzino-Demo, A., *Chemokines and defensins as HIV suppressive factors: an evolving story.* Curr Pharm Des, 2007. **13**(2): p. 163-72.
243. Lesner, A., et al., *A soluble factor secreted by an HIV-1-resistant cell line blocks transcription through inactivating the DNA-binding capacity of the NF-kappa B p65/p50 dimer.* J Immunol, 2005. **175**(4): p. 2548-54.
244. Tjernlund, A., et al., *Leukemia inhibitor factor (LIF) inhibits HIV-1 replication via restriction of stat 3 activation.* AIDS Res Hum Retroviruses, 2007. **23**(3): p. 398-406.
245. Triboulet, R., et al., *Suppression of microRNA-silencing pathway by HIV-1 during virus replication.* Science, 2007. **315**(5818): p. 1579-82.
246. Satterly, N., et al., *Influenza virus targets the mRNA export machinery and the nuclear pore complex.* Proc Natl Acad Sci U S A, 2007. **104**(6): p. 1853-8.
247. Pagtakhan, A.S. and S.E. Tong-Starksen, *Interactions between Tat of HIV-2 and transcription factor Sp1.* Virology, 1997. **238**(2): p. 221-30.
248. Ilyinskii, P.O. and R.C. Desrosiers, *Efficient transcription and replication of simian immunodeficiency virus in the absence of NF-kappaB and Sp1 binding elements.* J Virol, 1996. **70**(5): p. 3118-26.
249. Majello, B., et al., *Different members of the Sp1 multigene family exert opposite transcriptional regulation of the long terminal repeat of HIV-1.* Nucleic Acids Res, 1994. **22**(23): p. 4914-21.
250. Bargonetti, J., et al., *p53 represses Sp1 DNA binding and HIV-LTR directed transcription.* Cell Mol Biol (Noisy-le-grand), 1997. **43**(7): p. 935-49.
251. Maslove, D.M., et al., *Modulation of HIV transcription by CD8(+) cells is mediated via multiple elements of the long terminal repeat.* Clin Exp Immunol, 2001. **125**(1): p. 102-9.
252. Burnett, J.C., et al., *Control of stochastic gene expression by host factors at the HIV promoter.* PLoS Pathog, 2009. **5**(1): p. e1000260.
253. Weinberger, L.S., et al., *Stochastic gene expression in a lentiviral positive-feedback loop: HIV-1 Tat fluctuations drive phenotypic diversity.* Cell, 2005. **122**(2): p. 169-82.

254. Singh, A., et al., *Transcriptional bursting from the HIV-1 promoter is a significant source of stochastic noise in HIV-1 gene expression.* Biophys J, 2010. **98**(8): p. L32-4.
255. Verdin, E., P. Paras, Jr., and C. Van Lint, *Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation.* EMBO J, 1993. **12**(8): p. 3249-59.
256. Le Borgne, S., et al., *CD8(+)-Cell antiviral factor activity is not restricted to human immunodeficiency virus (HIV)-specific T cells and can block HIV replication after initiation of reverse transcription.* J Virol, 2000. **74**(10): p. 4456-64.
257. Belshaw, R., et al., *Long-term reinfection of the human genome by endogenous retroviruses.* Proc Natl Acad Sci U S A, 2004. **101**(14): p. 4894-9.
258. Garrison, K.E., et al., *T cell responses to human endogenous retroviruses in HIV-1 infection.* PLoS Pathog, 2007. **3**(11): p. e165.
259. Esnault, C., et al., *Dual inhibitory effects of APOBEC family proteins on retrotransposition of mammalian endogenous retroviruses.* Nucleic Acids Res, 2006. **34**(5): p. 1522-31.
260. Kinter, A.L., et al., *CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status.* J Exp Med, 2004. **200**(3): p. 331-43.
261. Eggena, M.P., et al., *Depletion of regulatory T cells in HIV infection is associated with immune activation.* J Immunol, 2005. **174**(7): p. 4407-14.